

nature

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Are scientists really stifling science?

SCIENTISTS are stifling science; Professor John Taylor of King's College, London put this proposition to an audience at the Royal Institution in a televised "Controversy" programme this week. Within the necessary confines of a televised debate it is all too easy to judge the matter won or lost on style rather than content; Professor Taylor raises an important issue and it merits careful consideration within the scientific community.

There is, of course, a very specific circumstance surrounding Professor Taylor's unease. For the past two years he has been attempting to come to terms with a variety of phenomena that are usually put into the paranormal category. His motive has, he insists, consistently been to explain these phenomena by means of conventional scientific concepts. History is on his side in this respect; many phenomena which in the past were regarded as bizarre or supernatural have yielded to scientific analysis. History, as his critics continue to urge (see Professor Hammerton's letter on page 640), is not on his side in offering too much encouragement that professional scientists can outsmart professional deceivers, if deceivers some be.

The issue at hand, however, goes far beyond metal bending. Scientists are in an unusually favoured position in policing their profession, and so they have a special responsibility to keep these policing mechanisms up-to-date, fair and seen-to-be-fair. Governments devote vast sums of money annually to the practice of science, and even when the science is obviously directed at solving pressing problems, the scientist is still the ultimate arbiter of what he does and doesn't do. Indeed through a variety of mechanisms from refereeing for journals to sitting on grant-giving bodies, scientists are arbiters of what others are allowed to publicise and spend money on. Do scientists warrant the trust that the public, unwittingly, puts in them to keep their house in order and forward-looking?

The greatest guarantee that the uncommon or unusual idea will get some sort of hearing is pluralism. If the sources of money are not controlled by just one committee, if the means of publication are not all in the hands of one person, if education is not according to some unified syllabus, then the prospect of any one group of people being able to exert an unhealthy influence is vastly diminished. And those who cannot get satisfaction through normal channels can often be remarkably effective in unusual ways. Research does

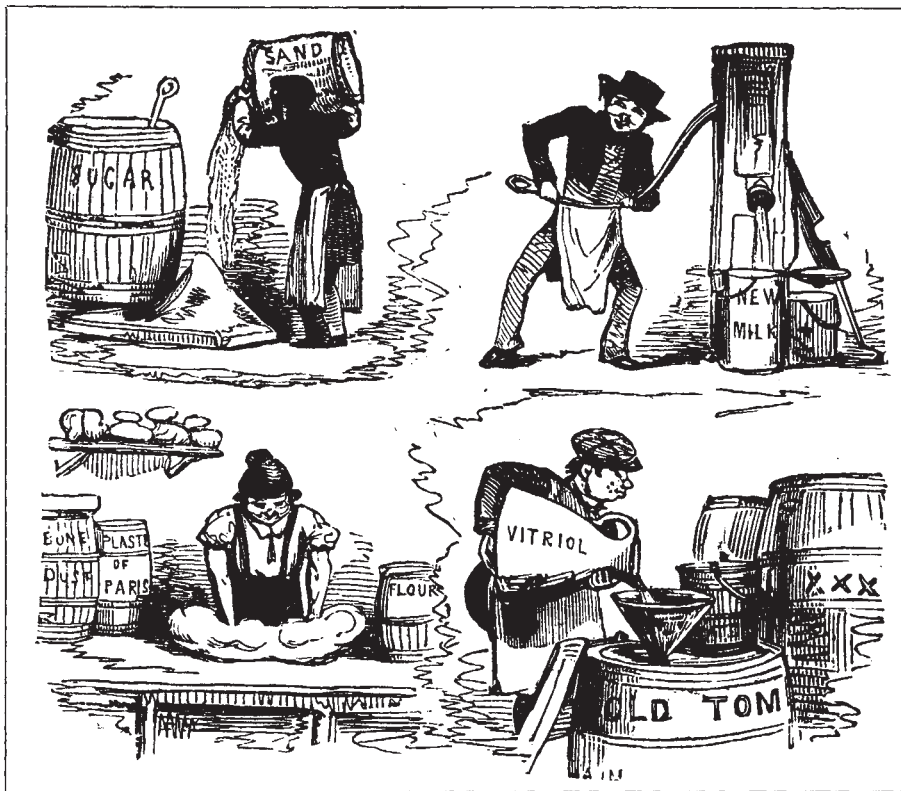
not have to have been sponsored by the Science Research Council to be distinguished, nor does it have to be published in *Nature* to command wide attention. Indeed some of those who feel they are unjustly ignored might be surprised how well known and extensively thought about their work is; simply because no-one relishes the thought of being known to posterity as the person who rejected a seminal idea. Likewise, as Professor Ball's letter (page 640) shows, experimental help does not necessarily come from a grant-giving body.

No doubt all of this sounds like smug self-satisfaction; the real test for the openness of the system, it will be said, is bound to be in the case histories rather than in assertions of fairness. The problem with case histories, however, is that conclusions have to be drawn from limited evidence and that there is often considerable difficulty in understanding the spirit of the time. Wegener's advocacy of continental drift in the early years of this century is often quoted as an example of an immensely important idea on which the establishment turned its back, thereby retarding the earth sciences by fifty years. But Wegener's ideas were well known and widely discussed; continental drift was the subject of at least one conference in the 1920s. Causes of drift were discussed extensively in the literature. What was missing was the compelling evidence that Wegener could not provide and that only an expenditure of large sums of money on marine research in the 1960s could. That example should warn us that certain fields can fail to flourish, even when confronted with an apparently blinding insight.

It should also warn that the case history is not an easy way of demonstrating inbuilt bias amongst scientists unless it is possible fully to appreciate the environment of facts, observations and speculations within which they lived at that time.

Science is ultimately about ideas moving around inside people. It does not reside in textbooks or in large pieces of equipment. Although it is possible for individuals to deny to other individuals the access to hardware or the literature through one particular channel, it is possible for no-one to control all channels, and it is certainly impossible to suppress ideas and people. This may be less true in other societies or in other professions, but surely the Western scientific community has had a relatively good record, and it is time that we stood up and said so. □





All the food that's fit to eat

During the latter half of the nineteenth century the public outcry about the adulteration of food in Britain led to the passing in 1875 of the first effective Sale of Food and Drugs Act. This Act, which is the basis of our present law, contained for the first time the requirement that all food sold must be of the nature, substance or quality demanded by the purchaser. It also required local authorities to appoint public analysts to check for purity samples of food, drink and drugs procured by local authority inspectors. To celebrate the centenary of the Act a symposium was held in London this week.

DURING this year's Annual Meeting of the British Association, Professor P. J. Newbould, of the University of Bradford, read a paper on the basic purpose to which the endeavours of modern science-based societies were directed. During mediaeval times, he pointed out, while nations struggled, as we do today, to be prosperous, the main aim of their citizens was salvation. In our own day it is economic growth and technological advance.

The symposium organised by the Ministry of Agriculture, Fisheries and Food to celebrate the centenary of the passing of The Sale of Food and Drugs Act 1875 was hardly intended to question that the continuous accretion of legislation and the elaboration of food standards and regulations, one following the other as analytical refinements and the minutiae of nutritional understanding advanced, is anything but good, nor that further control and standardisation in the future will be better. Indeed, the title of the symposium was "Food quality and safety—

a century of progress". Yet it is to be hoped that the sheer weight and size of the programme left time for someone to consider whether Harry Lauder's advice in the old song, to "keep right on to the end of the road" is always applicable. Perhaps, if we look around from where Professor Newbould is standing we may find that we are already approaching the end of one road and could usefully consider going in another direction.

In 1875, adulteration of food was rampant. Those were the days when "little drops of water, little grains of sand (made) the milkman merry, and the grocer grand". Something needed to be done to stop the watering of milk, the addition of alum to flour and even so flagrant a malpractice as the use of "sugar of lead", as lead acetate was called, to sweeten beer. Progress in analytical chemistry made it possible to police the 1875 Act. It is worth noting that the Society of Public Analysts, now the Society for Analytical Chemistry, was also established

in 1875 with Theophilus Redwood as its first president. The main purport of the Act was to decree that food on sale should be of "the nature, substance and quality" demanded. Provided his product came up to this straightforward standard, the merchant was, like other citizens of the realm, a free man innocently selling anything he pleased until he was proved guilty of marketing a product not of the "nature, substance and quality" his customer could reasonably expect. The march of time, the progress of science and the development of food technology in all its modern ramifications and, most important of all, a change in the philosophical outlook of the community, have led today to a radical difference in the approach of those who guide our society to what constitutes good wholesome food and the means by which these qualities can best be maintained. To start with, the analyst's ability to detect smaller and smaller traces of contaminants has enormously increased. Figures in terms of p.p.b. (parts per 10^9) are as commonplace nowadays as p.p.m. once were and for some compounds analysts can measure as little as 1 part in 10^{12} . Then again, the much simpler problem of determining whether a particular consumer had or had not suffered from poisoning by lead acetate immediately after having drunk adulterated beer is now replaced by the exceedingly difficult one of assessing whether, because a proportion of a colony of rats to which Butter Yellow had been administered developed cancer of the bladder, people consuming foods coloured with Butter Yellow would within a period of years extending up to 70 (it has only recently been decided that if the postulated harm is forecast as likely to occur later than this it can be ignored—after all, it does not much matter if you are poisoned after you are dead) be likely to suffer also. Nor could it be assumed that people who wished to buy a wrapped white sliced loaf, or mayonnaise from which an oily layer would not separate, or a jar of "coffee whitener", or a carton of soft margarine, or a can of luncheon meat knew any longer what "nature, substance and quality" it was that they were demanding—or so those responsible for the public welfare led the public to believe.

During the hundred years since 1875, the whole community comprising the ordinary citizen, the scientist, the diligent guardian of the public good and the food manufacturer have all been carried together along the stream of advancing technology. No longer do any of these consider that an ordinary person can be left to himself to recognise a sausage or a pot of jam. Step by step the law has taken it upon itself

to set up quantitative standards of composition, to lay down detailed specifications for what may appear on the label or in an advertisement. That is why it is forbidden any longer to sell a "digestive" biscuit, since the digestibility of all biscuits may be assumed, while Bristol Cream, deficient as it is in butter-fat content, retained its label by special dispensation. Only approved colours, preservatives, flavours and other substances added to improve the texture or retain the moisture—all so properly described as "additives"—can today be added. And behind all this stands the scientist who alone possesses the skill to determine whether the law has been obeyed and the rules kept.

Refinements of the present rules are coming as we watch. Already shopkeepers are being required on pain of prosecution to sell some foods before a predetermined date although so far it is not actionable to be caught eating stale bread. And the Food Standards Committee, recognising that beans are different from meat, are proposing that, should a manufacturer fabricate beans into the semblance and texture of meat, he should mix with them a due proportion of the amino acid methionine.

Clearly, it is important to insure that food is wholesome, uncontaminated by toxic substances and not misleadingly labelled. The question to be asked now, after a hundred years spent applying more and more scientific ingenuity, is whether the means have been justified by the ends that have been attained.

Have increasing health and happiness followed each addition of sophisticated control and regulation? Do labels bearing lists of "additives" in small print contribute to the contentment of those who eat baked beans, fish paste and tubs of margarine? The unification of standards of food composition and of the analytical procedure needed to verify their maintenance, first within the country as a whole, next throughout the European Economic Community and finally, as soon as can be done, across the whole world, is already in hand. A council of wise men such as that set up by James I to prepare an English version of the Bible is well advanced in its work to prepare the *Codex Alimentarius*, the world gospel of food composition.

Although it is right and proper for all these great men, distinguished in science and prominent in government and administration, nationally, internationally and world-wide, to review the progress that has been made, the end of a century of effort to apply science to the control of food quality and safety is a good time to stop, just as Professor Newbould has done, not

merely to see how nearly the target—of perfect quality and absolute safety—has been approached but to consider whether there is perhaps a different target to be shot at. Do people fuss and worry about the safety and wholesomeness of their food in spite of the devoted efforts of the Food Standards Committee, the Food Additives and Contaminants Committee and the Labelling of Food Regulations or because of them? Is the health of the community safeguarded more effectively by forbidding the use of cyclamates on the basis of subtle circumstantial evidence based on animal experimentation on which scientific opinions is, to say the least, divided or, let us say, by making cyclamates compulsory in order to minimise the consumption of sugar which is known to be harmful? There are other questions, likewise never asked, of which the biggest is: since, of all the contributing factors, lack of money is the most potent cause of poor diet, should we not measure the cost of the vitamin enrichment of bread and margarine, of analysis for the presence of required ingredients and the absence of proscribed ones, of the listing of components on labels, of the wrapping and packing of this and the discarding and disposal of that, to try to strike a balance between the benefits all these measures bring and the price that has to be paid for them.

These, however, are technical questions and, whatever shortcomings there still may be and whatever questions still remain unanswered, the distinguished people who set up the standards, do the analyses, measure the risks and add the vitamins are above all competent in their fields. The philosophy behind their century of forward march is, however, that of science and, as it sometimes seems, of a science that is assumed to be capable of ensuring that absolute safety and perfect purity—standards to which in the end all the world's food must conform. In the century ahead there is room for a new idea.

Obviously, our food must be fit to eat and we need to apply scientific understanding to keep it so. But, since there is no such thing as absolute safety, any more than there will ever be complete knowledge, there is room in the next century for those who invent the food laws to allow some of the people some of the time to eat at their own risk. Perhaps if the scientific advisers and governors let it thus be known that British scientists know that they do not know everything nor ever will and that they are not able to avert every "Act of God", the ordinary citizen would be less inclined to blame the government for every misfortune.

Magnus Pyke

One step from chemical automatons

by Akiyoshi Wada

By anyone's standards, the revelation of the molecular mechanism of life must be considered one of the highlights of the history of natural science. To make this revelation more meaningful and beneficial to mankind, those engaged in research on biological molecules must begin to think of possible practical applications of the molecular mechanism of life. For this purpose, a committee was formed in Japan about a year ago to examine and explore industrial applications of biological molecules with special emphasis on enzymes and other functional biopolymers. This is one of the projects under the auspices of the Council of Science and Technology in Japan for the promotion of life science. The committee is still carrying out its work, but wishes to present an interim report on the general image of what these applications might be, in the hope that it will stimulate a response from those who are engaged in biopolymer research.

A LIVING body is a molecular machine whose parts are molecules; machines using molecules as functional parts are obviously far more compact than present man-made machines. We already know the functions of individual parts, which make up complicated yet ordered chemical circuits. We also know how higher order structures are constructed and regulated, how energy is converted from one form to another, and how information is transmitted in these chemical circuits. The knowledge of these functions and mechanisms serves as the basis for the next stage of our project: the development of a chemical automaton with highly developed self-contained chemical circuits.

Rather like an electronic computer, this automaton would convert input into output; in the automaton's case, however, input and output would be molecules. An electronic computer is made up of hardware and software; a chemical automaton needs an additional component, a chemical reaction

system which might be called 'wetware'. The functional molecular units of a chemical automaton serve for:

- Reaction.
- Regulation.
- Information storage.
- Material transfer.
- Separation of materials.
- Transport of energy.
- Conversion of energy.
- Detection of parameters at each of the reaction stages.

The ultimate automaton is likely to be what might be called a 'synthetic living body' which consists mainly of wetware and software. But, for now we should concentrate on developing automatons which also include hardware such as measuring devices, computers and energy sources. For this purpose we must first develop the following topics:

- Stable and specific catalysis.
- Specific selective membranes.
- Reaction control systems including measuring devices.
- Chemical circuit theories.
- Energy transducers.

Topics in basic research which need to be studied and therefore to be supported for the development of the above items are:

- Biopolymers, in particular the structure and functions of enzymes.

- Molecular mechanisms of specific reactivity and its regulation.
- Organic reactions in aqueous and neutral conditions (without strong acids or bases).
- Synthesis of polymers of determined sequence.
- Stabilisation and immobilisation of natural enzymes for easy use.
- Specific permeable membranes.
- Mechanism of energy conversion in biological systems.
- Stability of chemical reactions in relation to spatial and temporal chemical oscillations.
- Development of highly sophisticated measurement techniques in physics and chemistry needed for the aforementioned research topics. (For example, we can cite the most up-to-date electronic and mechanical devices and computers.)

Many obstacles lie in the way of the realisation of chemical automatons. They may seem insurmountable, but in comparison with dreaming of flying 100 years ago, or speculating on high speed computers 50 years ago, chemical automatons are only a step away. In any event, nature provides chemical automatons as living bodies, and the principle behind their working mechanism is fairly well understood. Thus, the obstacles are indeed surmountable,

and efforts made in overcoming these obstacles are well worth exerting, when we think of the beneficial effects of chemical automatons on society.

Some of the direct effects would come from a new form of chemical industry, operating at normal temperature and pressure in the state of aqueous solution, requiring minimal energy and producing no harmful by-products. Other benefits would be flow production of complex chemicals which does not disturb the environment, and the possibility of the full use of substances abundantly available in nature, such as air, water and carbon dioxide.

Indirectly, chemical automatons would affect both basic and applied sciences covering a wide range of fields such as biophysics, molecular biology, biochemistry, polymer chemistry, the textile industry and the pharmaceutical industry.

In the advanced nations, including Japan, the potential for the development of chemical automatons is great. Barriers do, however, exist for such people to switch their specialities to life science. The automaton project straddles over many academic disciplines, and it is hoped that it could serve to trigger meaningful interdisciplinary research centring on the molecular life sciences. □

international news



Cornforth: shares chemistry prize.

Eight share Nobel Prizes

DURING the past week, Nobel Prizes for medicine, chemistry and physics, at £69,000 tax-free a time, have been shared by eight European and US scientists. The prize for medicine was shared by Renato Dulbecco, a 61-year-old Italian-born American now working at the Imperial Cancer Research Fund, London, and two of his former pupils—David Baltimore, aged 37, of the Massachusetts Institute of Technology, and Howard Temin (40), of the University of Wisconsin.

Baltimore was Dulbecco's student at the Salk Institute for Biological Studies, and Temin studied under him at the California Institute of Technology. They receive their prize for their work on the circumstances and effects which cause cancer. Dr Dulbecco's work has been on the mechanics by which animal viruses insert their own genetic material (DNA) into that of

animal cells. This can enable them to convert the cell into a virus factory, but it can also transform the cell into a cancerous state.

Dr Temin and Dr Baltimore share the credit for an outstanding discovery about RNA tumour viruses. RNA usually acts as the messenger molecule by which the genetic instructions of body cells are translated into proteins. Dr Temin and Dr Baltimore were convinced that RNA tumour viruses must be able to reproduce themselves in a DNA form to enter the cell's genetic machinery. Ten years after Dr Temin first predicted it, both men almost simultaneously found the enzyme that enables them to do it. The enzyme enables the virus to make DNA from RNA.

The discovery stood conventional biology on its head because it reversed the genetic process by which DNA makes RNA and RNA then makes protein.

Many RNA viruses are involved in animal cancers and it is believed that some human cancers may be caused in the same way. Work with these viruses

has also shed considerable light on the basis of the genetic workings of cells.

The chemistry prize was shared by John Cornforth (58), now at the University of Sussex, and Vladimir Prelog (69), a Yugoslav-born Swiss citizen who teaches organic chemistry at the Federal Institute of Technology in Zurich. The Royal Swedish Academy of Sciences said that Cornforth, who has been deaf since childhood, had made "an outstanding intellectual achievement" in his work on the stereochemistry of enzyme-catalysed reactions.

Professor Cornforth has used enzymes, which are natural biological catalysts for speeding chemical reactions, to look at the synthesis of biological molecules. In particular, he has shown the steps by which cholesterol is made.

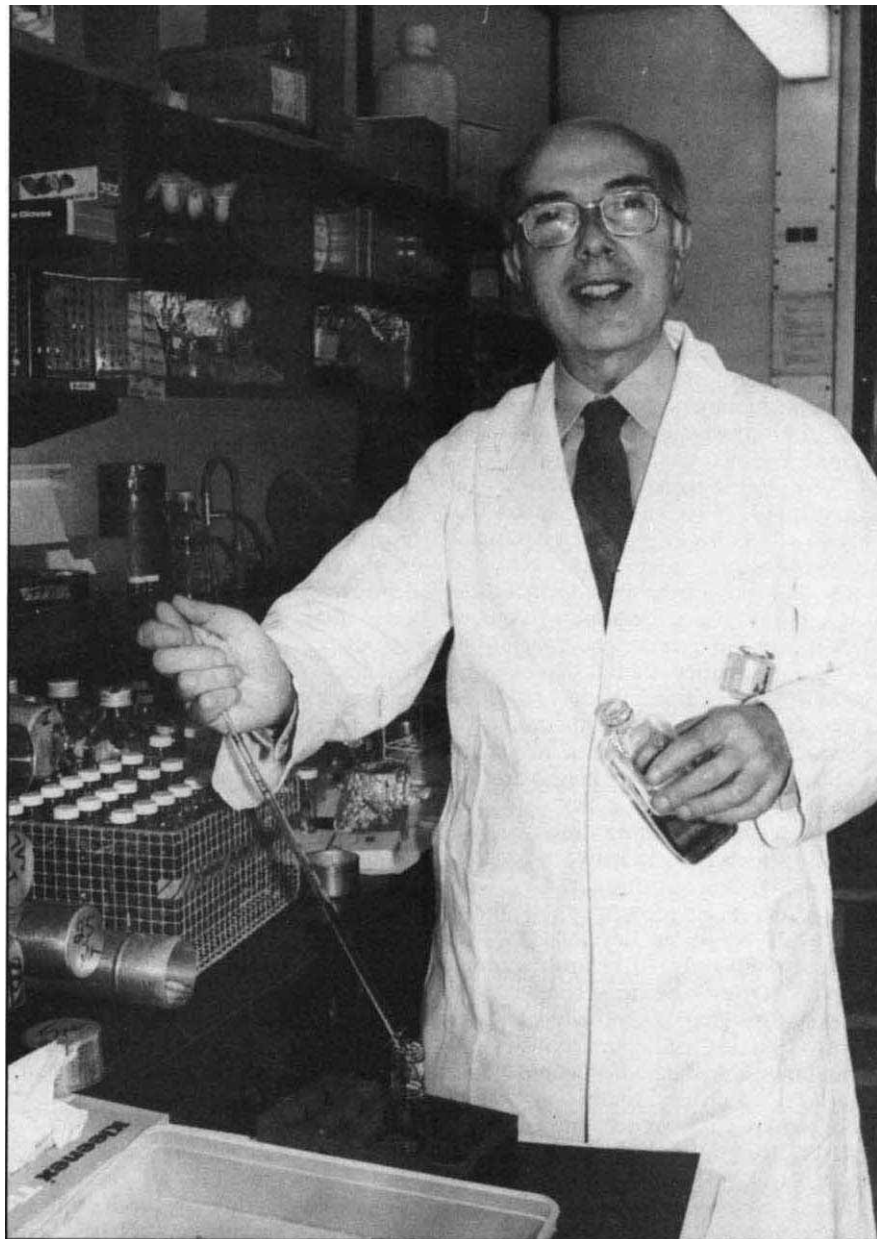
Dr Prelog has contributed to an understanding of how natural molecules are often produced in an asymmetrical fashion. Many molecules can exist either in a "right-handed" or in a "left-handed" form. Artificial manufacture of such molecules generally produces equal amounts of each. But in nature, only one is produced.

An American and two Danish nuclear scientists shared the physics prize for research into the structure of atoms. They are James Rainwater, of Columbia University, New York; Aage Bohr, of the Niels Bohr Institute, Copenhagen; and Ben Mottelson, of Nordita, Copenhagen.

Rainwater, Bohr and Mottelson won their prize for the invention of the 'collective' model of the nucleus. This is a synthesis of two earlier models: the liquid-drop model (a rather coarse, very 'collective' model in which the nucleus is pictured as a charged drop which deforms as a whole; it was very successful in explaining the masses of nuclei and fission) and the shell model (in which nucleons are pictured as existing in individual orbits and forming closed shells; this leads to the idea of 'magic numbers'—particular numbers of nucleons conferring exceptional stability on the nucleus).

The latter model was much more detailed in its treatment of the nucleus, and one could derive much more information about excited states from it. Experimental deficiencies were observed, however; many nuclei exhibited much greater quadrupole resonances than could be explained on the shell model, and also some nuclei showed an excited level structure reminiscent of rotational bands. The collective model pictures the outer nucleons, in individual orbits, interacting with the closed inner shells and deforming them—this gives the 'collective' effect, and achieves the synthesis of the two models. □

Dulbecco, pictured at ICRF this week



Howard Temin



David Baltimore

Education issues in Japan

ONE of the most commonly quoted statistics about Japanese higher education is that graduates comprise only about 3% of total student numbers (compared, say, with the UK where graduates comprise 20% of all students). What is less often mentioned is that even this 3% meant 46,000 graduate students in 1973—more than in the UK. Even so, the concept of the graduate course as an integral part of the structure of a university is less common in Japan. As the table shows, half of all universities do not have such courses.

Since the Second World War the number of universities in Japan has increased enormously. For national universities the government took the view that newly established institutions should not have graduate courses; rather, the courses in the former imperial universities should be enlarged in size and scope. Some additional financial support was forthcoming for research and education, but in general staff numbers and facilities were not allowed to increase.

This selective funding, however, was seen to widen the gap between research activities in universities with and without graduate schools, so great, almost fanatical, pressures developed in the early 1960s to develop graduate courses. Originally such courses were intended primarily to educate new research scientists, but industry increasingly demanded people with master's degrees, many of which courses are now much more closely related to production processes than to research.

The market for people with doctorates is limited to universities and the research institutes of government and private industry. But enrolment in higher education seems to be reaching saturation, so universities are no longer taking on many new staff; the same is true of most other institutions, with exceptions only in the environmental and social sciences. The problem of the so-called 'over-doctors' is serious, and many have proposed an increase in the number of postdoctoral fellowships as a way of preserving a reservoir of highly qualified manpower for future demands.

The Japanese concept of the chair (*koza*) undoubtedly had its origins in the European professorial chair, although there are many differences.

It is the structural unit of the faculty, responsible for education and research in a specific subject area, and is composed basically of one professor, one assistant professor and two assistants. It is provided with its own running expenses and facilities. Although often criticised as an indestructible castle of conservation and hierarchy, it functioned reasonably well in the pre-war imperial university, and has also been fitted into the present frame-work of graduate education. Partly because of the rapid expansion of the size of enrolment, and partly because of the enormous development of the sciences, the idea emerged that it would be more desirable to lift the restriction that the structural unit for education should be identical with that for research. It was hoped that this would make teaching as well as research more flexible and dynamic. After lengthy discussions, the laws governing the structure of the universities, both national and private, have been modified to allow the possibility of avoiding the traditional structure of the faculty based on the *koza* system, and to admit a more flexible one. Any new venture has to be discussed with the Ministry of Education, of course.

One of the issues from a recent report from the University Council (*Daigaku-setchi Shingikai*) in the Ministry of Education is the possibility of independent graduate institutions outside the universities. Although apparently a reasonable proposal, and even though such an institution may be highly qualified in research, there still needs to be an appropriate mechanism to link research with the educational background characteristic of a university. The lack of a mechanism for encouraging interaction between research and education could be harmful. The report also mentioned that it would be worth considering associated graduate institutions administered through the cooperation of several universities. Serious efforts are being made in certain groups of universities (including cooperation between national and private universities) to encourage the exchange of teaching staffs or of student credits. These changes will be particularly useful in developing interdisciplinary courses such as information science or environmental science.

from Yoshinoku Kakiuchi, Tokyo

	Total no. of universities	master's degrees only	Universities offering	
			master's degrees and doctorates	doctorates only
National	81	38	26	1
Public	33	4	8	7
Private	299	37	72	12
Total	413	79	106	20

Anti-nuclear critic faces dismissal

by Allan Piper

A LEADING West German nuclear scientist is faced with dismissal from his chair at the University of Bremen, apparently because of his support for the anti-nuclear energy lobby. Dr Jens Scheer, Professor of Nuclear Physics at Bremen and a state parliamentary candidate for the German communist party (KPD), has claimed that the action against him arises out of his active criticism of the West German nuclear programme, and that it is not a consequence of his political affiliations, as is officially claimed.

Professor Scheer was suspended on September 23 following his alleged involvement in a disturbance on the university campus during which the officials of a civil court were attacked by egg throwers. Though he denies the charge he will now face a special disciplinary tribunal. The University authorities have stated that they are seeking his dismissal, the official reason given being that Professor Scheer, as a member of the KPD, has contravened "the code of honour which every [civil servant] must, through his whole behaviour profess and seek to maintain". Although as head of a university department Professor Scheer holds what is effectively a life appointment under existing German law, his dismissal on those grounds would be perfectly in order. A growing body of support for him maintains, however, that a little used legal clause has been invoked to mask the real reason for dismissal.

Professor Scheer's supporters, including a large number of eminent European academics and political organisations, believe that in seeking to remove him from his position at the university, the authorities hope to overcome a potentially powerful source of opposition to the West German nuclear energy programme. As yet Germany has no nationally coordinated anti-nuclear lobby but in the past Professor Scheer has often provided expert support for small, local protest organisations. Further, since his arrival at Bremen in 1971 Professor Scheer has established his department as a centre for the study of the interactions between science, technology and society, and in May this year the department published a book examining the strategy and consequences of nuclear development.

● Yesterday it was learnt that Professor Scheer's suspension has been overruled by a court of law, allowing him to resume his normal duties at the university, but dismissal proceedings will still go ahead. □

AN international group of scientists involved in bacteriophage work is unhappy about the way in which the Recombinant DNA Molecule Program Advisory Committee is handling the problems of plasmid engineering, and a meeting of the committee planned for this month has been put off until December because of the concern which has been expressed about its last meeting at Woods Hole.

The group, which consists of 48 scientists who attended the recent Cold Spring Harbor bacteriophage meeting, has sent a petition to the National Institutes of Health (NIH), complaining that the Woods Hole guidelines on recombinant DNA represent a watering down of the recommendations made at the Asilomar conference earlier this year. The Asilomar conference, convened by Professor Paul Berg, was the first gathering of workers in the field to discuss the potential dangers of plasmid engineering, and a subsequent meeting of UK workers at Oxford broadly confirmed the working rules suggested at Asilomar.

In a letter to Dr Dewitt Stetten, Deputy Director for Science, Office of the Director, NIH, the Cold Spring Harbor people complain that a draft of the Woods Hole meeting called "Current Guidelines for Research on

Recombinant DNA Molecules" appears "to lower substantially the safety standards set and accepted by the scientific community as represented at the meeting at Asilomar in February, 1975".

DNA committee has its critics

The letter "strongly requests" that the advisory committee considers at its postponed meeting the feelings of the group in three areas.

- They urge that the most hazardous experiments be curtailed until some experimental determination of the risks inherent in such procedures is made. They say, for instance, that the extent of containment possible with different vectors remains to be shown.

- They are concerned that any mammalian DNA (let alone animal viral DNA) can, by the present draft, be cloned under less than P3 containment, and they say that they are not persuaded that an untested vector designed for safety reasons is by itself an adequate safeguard for such experiments in an open laboratory. They add that "strong consideration should be given to limiting shotgun experiments of mammalian DNA to P4 contain-

ment until proven safety vectors are available".

- They feel that the composition of the committee should be broadened to include more representation from the areas of animal virology, plant pathology and genetics, and epidemiology; and also that the advisory committee should have much stronger representation from scientists not directly involved in cloning experiments. And taking a line which the committee will surely find hard to swallow, they think it advisable "to consider representation of the public at large".

One of the organisers of the petition, Richard N. Goldstein, of the Harvard Medical School, says the letter "reflects a deep concern" with the results of the Woods Hole meeting, and he believes it is "exceedingly important that the general scientific community be made aware of these developments".

- This week Dr Goldstein reported that the Woods Hole guidelines had been scrapped. According to Goldstein, Dr Betty Kutter, "a vocal critic of these guidelines and a member of the Recombinant DNA Molecule Committee, has been charged with the re-writing of these guidelines as a result of the pressure put on the committee by many dissatisfied scientists".

EVER since the days when medicine was largely the province of hucksters and snake oil merchants, people have been touting cures for cancer. Nowadays, such cures may be mentioned briefly in a racy tabloid newspaper, but they are usually ignored by self-respecting scientists, attract mercifully little following, and are quickly forgotten. But not so with one purported anti-cancer remedy called Laetrile.

Although declared contraband by the federal government, outlawed by several state governments and found to be utterly worthless in a number of tests carried out at several prestigious cancer research institutes, Laetrile is now being consumed by an estimated 20,000 people in the United States. It is available on the black market, or through clinics in Mexico and West Germany, to which desperate American cancer sufferers are flocking in droves. It owes its popularity in the United States to a vocal, and at times heated, campaign by a number of groups on the West Coast who are fighting to get legal restrictions on Laetrile lifted.

The bitter battle over Laetrile would have all the ingredients of a good thriller, if the subject matter were not so tragic. Research reports have been stolen and given wide publicity, an international smuggling ring has been broken up by federal agents, cancer

Trials for Laetrile

by Colin Norman, Washington

researchers have been accused of deliberately suppressing information, numerous court fights have occurred, and right-wing political groups have been accusing the government of invading personal freedom. The matter has certainly caused a headache for the Food and Drug Administration and the National Cancer Institute, and a good deal of embarrassment for the Memorial Sloan-Kettering Cancer Center in New York.

Laetrile was apparently first used for cancer treatment in the 1920s by a California doctor called Ernest T. Krebs, Sr, but it was too toxic to be much use. A purified form was developed in 1951 by Krebs' son, E. T. Krebs, Jr, a biochemist, who claimed that the substance was safe for injection. More recently, Laetrile has been produced in a form which can be taken orally, and its use has skyrocketed.

There have been numerous anecdotal reports of cancer sufferers who have gone into remission after taking Laetrile, or who have at least experienced a cessation of pain and have died in relative peace. But there have been no formal, clinical trials to test the efficacy of the substance, and until recently

there have been few animal trials to test Laetrile's purported anti-cancer activity. Results of two extensive animal trials will, however, be published later this month. They are unambiguously and crushingly negative.

Proponents of Laetrile have even suggested an elaborate mechanism to explain its alleged action. The substance, they suggest, is broken down inside cancer cells by the enzyme β -glucosidase, to release benzaldehyde and hydrogen cyanide in sufficient quantities to kill the malignant cells. Normal cells, they suggest, are protected because they contain the enzyme rhodanese which, in the presence of thiosulphate, converts hydrogen cyanide to the less toxic thiocyanate. It is a neat mechanism which every cancer chemotherapist looks for—something which is entirely specific to cancer cells but non-toxic to normal cells. The trouble is, though, that there is not a shred of evidence so far to support it.

The campaign in support of Laetrile certainly has considerable popular appeal. A film developed for the pro-Laetrile forces, for example, begins with the following statement: "This year, 250,000 Americans will die from cancer ... this great human tragedy can be stopped now entirely on the basis of existing scientific knowledge". It goes on to note that "the history of science is the history of struggle

against entrenched error", and that some of the greatest scientists were ridiculed in their own time. It states that "with billions of dollars spent each year on research, with other billions taken in from the cancer-related sale of drugs, and with vote-hungry politicians promising ever increasing government programs, we find that, today, there are more people making a living from cancer than are dying from it", and argues that scientists therefore don't really want to find a cure for cancer because "if the riddle were solved by a simple vitamin [Laetrile], this giant commercial and political industry could be wiped out overnight".

In 1972 and 1973, the campaign took a new tack. Petitions, said to be signed by 43,000 people, were sent to former President Nixon demanding that clinical trials be run on Laetrile to test its anti-cancer properties. The petitions were responsible for launching four animal studies: two were carried out under contract to the National Cancer Institute (NCI), and two others were performed at the Memorial Sloan-Kettering Cancer Center and the Catholic Medical Center in Queens, New York.

Results of the two NCI studies will be reported later this month in the September/October issue of *Cancer Chemotherapy Reports*, a journal published by the NCI. Conducted by Isidore Wodinsky and Joseph Swiniarski at Arthur D. Little Inc. (ADL) in Cambridge, Massachusetts, and by W. R. Laster, Jr, and F. M. Schabel, Jr, at the Southern Research Institute (SRI) in Birmingham, Alabama, the studies involved large doses of Laetrile administered to mice bearing transplanted tumours. A standard method of screening for anti-cancer drugs, it consists of transplanting pieces of tumour from one mouse into other, genetically identical, mice and observing the effects of drugs on tumour growth. The ADL studies involved four different types of cancer, and the SRI tests three.

According to Wodinsky and Swiniarski, Laetrile did not prolong the life span of mice bearing any of the transplanted tumours, even at doses close to the level at which the substance was found to be toxic. They therefore conclude that "no anti-tumour activity was found in any of the four systems tested". Similar results were reported on the basis of the SRI tests. Laster and Schabel conclude that Laetrile "did not demonstrate significant anti-tumour activity against any of these three tumour systems".

Such negative results would normally be sufficient to close the book on a possible anti-cancer agent, but not Laetrile. The studies at the Sloan-Kettering have, inadvertently, provided

some ammunition for the pro-Laetrile forces.

According to Dr C. Chester Stock, a vice-president of Sloan-Kettering, and director of the institute's Walker Laboratories, his institute took a brief look at Laetrile in the early 1950s, but found no effect when it was tested against one implanted tumour system. The work was then dropped because "other agents were showing promising activity", he said. But when the petitions started flooding into the White House and letters began arriving at the Sloan-Kettering, demanding that Laetrile be tested, it was decided that the institute should take a second look at the substance. Particularly pertinent to that decision was the fact that the chairman of the President's Cancer Panel, Benno C. Schmidt, is also a board member of the Sloan-Kettering Institute (SKI). According to Stock, tests were run on "a battery of transplanted tumours" at high doses, in 1972, but he said "we didn't see any benefit whatsoever"—Laetrile again proved to be ineffective as an anti-tumour agent.

Although the use of transplanted tumours is a standard procedure for testing anti-cancer drugs, it has recently come under considerable criticism on the grounds that transplanted tumours may not behave like spontaneously occurring ones. The decision was therefore made at the SKI to test Laetrile on a strain of mice (developed by Dr Daniel Martin at the Catholic Medical Centre) which has a very high incidence of spontaneous mammary tumours. The tests were begun in 1972 by one of the SKI's most venerable researchers, Kanematsu Sugiura.

Sugiura's initial studies seemed to show a surprising effect. Although Laetrile apparently had only a small and variable effect on the primary tumours, it seemed to have a marked effect on the appearance of lung metastases. According to tests run between September 1972 and June 1973, Sugiura found that whereas 78% of the mice in control groups had lung metastases present, only 17% of those treated with Laetrile developed metastases. Sugiura wrote up the results of his experiments in a report which he circulated to his colleagues.

In the autumn of 1973, somebody at the SKI sent a copy of Sugiura's report to Laetrile proponents on the West Coast, and it subsequently got banner headline treatment. Supporters of Laetrile began to claim that the prestigious Sloan-Kettering Institute had proven the effectiveness of Laetrile, and that the institute was suppressing the results.

Since that time, Sugiura has continued to study the effect of Laetrile

on spontaneous mammary tumours in the mice bred by Martin, and parallel tests have been run by two other researchers at the SKI and by Martin himself. Sugiura has continued to find that Laetrile seems to inhibit the formation of lung metastases, but the other three investigators have all come up with negative results. Reports of Sugiura's recent research have been circulated within the SKI, and a month or two ago they were again leaked to Laetrile proponents on the West Coast, who distributed them widely to the general press.

In view of the wide publicity which the studies have received, and the charges that the SKI has been suppressing results favourable to Laetrile, Dr Stock recently discussed the issue.

Stock said that "because of the emotion surrounding this issue, we felt that we should be cautious". It was agreed that Sugiura and Martin should cooperate in a test on Laetrile in mid-1974. The experiment proceeded in exactly the same manner as Sugiura's earlier studies, except that lungs from the mice were subjected both to microscopic examination and to bioassay—the lungs were transplanted into a fresh animal to see whether tumours grew. Bioassay provides an objective assessment of whether or not lung metastases were present. The results proved negative—there was no difference between mice treated with Laetrile and the controls. Similarly, Stock said that the two other SKI studies proved to be negative.

Thomas emphasised that the SKI has certainly made no attempt to suppress results favourable to Laetrile, and he said that "I think it is safe to say that if it had been any other agent under study, we would have lost interest in it" when Sugiura's early results seemed to indicate that it had little or no effect on primary tumours.

The fact remains, however, that in spite of a large, and growing, body of information which indicates that Laetrile has no anti-tumour effect in animal systems, the substance is continuing to grow in popularity, and its proponents remain convinced of its efficacy. They are still demanding that human trials should take place.

Most cancer specialists are adamant that clinical trials are certainly not justified. Thomas, for example, said that "in the absence of positive data from animal experiments, use of Laetrile in human beings with cancer remains extremely difficult to justify, particularly in patients with cancers that are treatable by surgery, radiotherapy or chemotherapy". The Food and Drug Administration (FDA), which would have to licence any clinical trials, agrees. An FDA official pointed out that it would be difficult to

It is not, I hope, a serious contravention of the Official Secrets Act to reveal that, in Committee Room 11 in the Houses of Parliament, the wall above the rostrum is adorned with an enormous oil painting which depicts "The Speakers Procession in 1884". The Parliamentary and Scientific Committee sometimes meets in this room, and when the proceedings are less than enthralling I find myself studying the picture. My interest is not so much in attempting to identify the impressive figures (the print on the key diagram is too small to be read at a distance) as in wondering at the appalling mess of litter and waste paper through which the procession is wading. It is almost like the platform of a London underground railway station in 1975. I am glad to say that the corridors of power are, in this respect, more hygienic today, if other places are becoming filthier.

Litter and refuse are undoubtedly providing some of our most difficult pollution problems today. Both New York and London seem to be permanently subject to piles of stinking refuse, even when there is no industrial dispute between the local authorities and the refuse collectors. Earlier this year the strike of dustcart operators in Glasgow faced the city with a serious public health problem, so that the army had to be called in to clear away the more noisome dumps. The problem is made worse by the way in which the bulk of the litter is swelled by masses of unnecessary packing material, but its danger is also a function of affluence, for it is the waste food, much of it initially quite fit for consumption, that makes refuse a public health problem, attracting rats and increasing the risk of transmission of disease.

What may not be generally realised is that it is the successful control of air pollution which exacerbates the problem. Earlier this century the volume of refuse was much smaller, partly because much was burned on domestic grates and kitchen ranges.

It is true that some of the more

affluent do have refuse disposal units, to pulverise food and some other wastes, which are then discharged into the sewers. This may allow their hygienic disposal, though there have been reports from America of a consequent overload of sewage works and even more serious pollution of rivers when the material is discharged, untreated, into them. We are often better at moving pollution from one place to another than in curing or controlling it.

The Chief Scientist to the Ministry

Waste line



KENNETH MELLANBY

of Agriculture, Fisheries and Food, has recently stated that, of the food bought in Britain, as much as a quarter may be wasted. This does not include the substantial amount that is eaten, over and above their actual requirements, by most of our population, contributing to the obesity which is the most serious symptom of malnutrition in most Western countries today. It seems likely that, if we eliminated waste and gluttony, we could reduce our balance of payments deficit by nearly £1,000 million, without any marked change in our feeding habits.

Although there is a growing interest in recycling all types of waste material, little food is re-used in this way. In the past, much pig swill came from hotels, schools and even from private houses. Since swine vesicular disease has

become a serious problem, such collections of swill have greatly decreased, as regulations to ensure that the food is properly treated and sterilised, though wise and necessary, are too stringent for the majority of small operators. It is calculated that the dustbins of Britain contain at least 3 million tons of "putrescible material" (the trade jargon for waste food) a year, much with a high protein content, and that if this were properly treated it could feed more than 1 million pigs—as well as reducing the health risks arising from uncollected refuse.

If waste food is to be used to feed animals, it has to be separated and treated. There are less difficulties in recycling refuse as a soil fertiliser or conditioner. Considerable amounts of sewage sludge are used in this way, and several cities have installed elaborate and expensive plant to produce municipal compost. Wastes brought in by the dustcarts are sorted and separated (tins and iron scrap is removed magnetically, baled and sold) and the remainder, with added sewage sludge, goes through a digestive process. The end product is a brown powder which is quite pleasant to handle. It contains a significant amount of nutritive salts, and is an excellent conditioner for intractable clay soils. Although available at a low cost, most farmers have been reluctant to use municipal compost, because it usually contains substantial amounts of heavy metals, the lead level commonly exceeding 200 p.p.m. There is evidence that, in the presence of a high level of organic matter, this lead is hardly taken up by most plants, but it does remain in the soil and might give rise to problems at a later date if the organic level fell substantially.

Waste disposal and re-use is thus seen to be a very complicated problem. Clearly the best solution is to produce less, and to see that what cannot be avoided does not give rise to further problems. But it is at least encouraging that our present Members of Parliament are better house trained than their Victorian predecessors.

design an acceptable clinical trial without depriving patients of other, accepted methods of treatment. In other words, if Laetrile were put on clinical trial, it could only be justified ethically if it were simply added to other forms of treatment, and that wouldn't make for a very rigorous test.

There is, however, another extremely important aspect to the Laetrile debate. Laetrile, in its purified form, is relatively non-toxic. It can be fed to animals in large doses before any adverse effects are noted; according to Thomas, at least one of the "solid

pieces of data" to come out of the SKI studies is confirmation of that fact. Laetrile proponents therefore argue that FDA should not regulate the substance as a drug, but as a food or even a vitamin.

Would it do any harm if the FDA were to allow Laetrile to be marketed like any other so-called health food? Even the *New York Times* has suggested, in an editorial published in August, that since Laetrile is usually taken by cancer patients who are beyond help from conventional therapies, they should not be denied even a

possible placebo effect from the substance. But a letter from Dr Sherwin Gardner, Deputy Commissioner of the FDA, took exception to that suggestion. Pointing out that the FDA has a duty to protect the public against ineffective drugs, Gardner said he believes that "the idea of fraudulent promotion and sale of bogus cures to the desperately ill and dying is appalling". It is also argued that, if Laetrile is made more widely available, it will be taken by cancer patients in preference to other therapies which have at least proved effective in the past. □

correspondence

Laboratory charges

SIR,—I am much in sympathy with Kenneth Mellanby (October 2) and especially his final paragraph on inexpensive biological research. Having simple research needs—eyes, a waterproof and time for fieldwork and contemplation—I have been conscious throughout my career as a littoral ecologist, of the patronising designation 'only a naturalist' from those who think that little of value can be achieved without, for example, an autoanalyser, an atomic absorption spectrophotometer or an electron microscope. Tempted though I have been at times to try to impress visitors with a benchful of borrowed equipment and some coloured liquids their attitude has nevertheless remained as water on a limpet's back, and the major events of recent years have finally reinforced my conviction that a field of biology that is important but relatively inexpensive, that appears simple but is very challenging, has been ignored in favour of the esoteric and the elaborate.

This event is the 'ecological crisis', and more particularly within my own discipline the dawning realisation that by generally eschewing organismal biology for several decades marine biologists are not now able to tell society with conviction the state of affairs in the sea or along the coast. Time after time one is asked if pollution is affecting local populations, if a scarcity of this or an abundance of that is natural or not. With embarrassment one admits to ignorance, except in obviously severe instances, and then, in response to mutterings about taxpayers' money and what biologists have been doing all these years, one loyally defends colleagues who did not find it worthwhile intellectually or in career terms to count organisms, or to study their biological interactions, their reproductive cycles, their behaviour or even how long they live—all of which would have better enabled us to assess the present field situation and respond to the public concern. But attitudes are changing; the doom-mongers have, after all, done biology a service.

To those who may wonder what to turn to if their capital investment should be rendered valueless by an inability to meet the unforeseen but swingeing maintenance contracts, may I offer a suggestion? Lend a hand on a field course and encourage accurate

observation and speculation about what organisms do, how they live in a complex natural situation. The range of as yet unanswerable questions about even the most common species that will surely arise from a class of enquiring minds will not only be humbling but will point to many rewarding research projects. In short, natural history appears in many up-dated guises but still presents unlimited and relevant challenges.

Yours faithfully,

J. R. LEWIS

*Wellcome Marine Laboratory,
University of Leeds, UK*

This week's free offer

SIR,—In a recent BBC "Controversy" programme, Professor John Taylor expressed strongly his opinion that scientists were stifling science. One of his points of contention was that he had been unable to obtain a grant from the Science Research Council to support investigations into the supposed paranormal phenomena associated with Uri Geller and others, including some children. In discussion he said that he had not the facilities for a thoroughgoing scientifically controlled experiment and mentioned that these could include such expensive items as electron microscopes and audiovisual recording equipment.

The phenomenon most frequently mentioned is the deformation and fracture of metallic objects without the application of the relatively large stresses normally to be associated with such changes in the metal shape. If it were true and could be substantiated, this would be an entirely new metallurgical phenomenon.

The equipment required for its investigation, including all the items mentioned by Professor Taylor, is available in this department and could be used for a definitive experiment. I would therefore like to invite Professor Taylor to join with us in devising a controlled experiment which would test the authenticity of the claims made by those, and particularly Uri Geller, who say that they can bend metallic objects without the direct application of the stresses normally required.

I would suggest that to establish the experiment's authenticity, its control and observation by independent witnesses should be approved beforehand by yourself and/or a nominated panel

of scientists and conjurers. The experiment could be devised to be definitive and its cost would be very small in relation to the importance of the claims that have been made. Professor Taylor need have no recourse to financial resources as the conduct of the experiment would be within the normal research activities of a department such as this. I very much hope that Professor Taylor will be willing to take up this proposal.

Yours faithfully,

J. G. BALL

*Department of Metallurgy and
Materials Science,
Imperial College, London, UK*

Cheating children

SIR,—Professor Taylor has cited a remark of Sir William Crookes on the relative susceptibility to deception of physicists and conjurers. It would materially help in weighing this statement if he would tell us whether it was uttered before or after Sir William had disgraced the scientific calling by becoming an accomplice of the fake medium Eliza Cook; if the latter, was it before or after the conjurer Maskeyne indicated the mechanism of deceit?

Yours faithfully,

M. HAMMERTON

*University of Newcastle upon Tyne,
UK*

Out of town

SIR,—May a humble linguist point out that "commanded out" ("Publishing problems", September 4) is probably an attempted translation of the German *abkommandiert*, which is a military (and probably bureaucratic) term meaning "temporarily transferred" to another place ("detached" is the English term as opposed to a permanent "posting"). Central Europeans, particularly scientists and technologists, often use German as a lingua franca; and central Europeans tend to be rather 'disciplined' in their jargon as opposed to the trendy Anglo-American 'free-and-easy' style. It is all a matter of custom. Without prejudice to your correspondent's point, his friend may merely have meant to say "I was out of town for a couple of weeks, and couldn't reply earlier".

Yours faithfully,

A. LODGE

Swanley, Kent, UK

news and views

FOR over a decade scrapie has excited the interest of scientists in all parts of the world because of the unusual nature of the disease and its causal agent; few diseases have aroused so much discussion and speculation.

Originally scrapie was studied as an important disease of sheep which, after a very long incubation period, can be recognised by the characteristic signs of uncoordinated movement and "scraping" or rubbing of the skin. These signs are associated with damage to the brain and, under the light microscope, the brain lesions appear mainly as vacuolation. It is now recognised, however, that sheep scrapie is the best known member of a larger group of diseases that includes transmissible mink encephalopathy (TME) and the two diseases of man, kuru and Creutzfeldt-Jakob disease, all of which have a similar brain pathology. Some recent studies of scrapie and TME in golden hamsters have shown that these diseases are almost indistinguishable either histologically, biochemically or biologically when compared in a common experimental host (Kimberlin and Marsh; Marsh and Kimberlin, *J. infect. Dis.*, **131**, 97-103, 104-110; 1975). It now looks as though scrapie may be important in studies of an even wider group of diseases because some strains of the scrapie agent can produce cerebral amyloid deposits in mice which are broadly similar to the amyloid plaques seen in aged human beings and in some neurological disorders of man such as Alzheimer's disease and Down's syndrome (Bruce and Fraser, *Neuropathol. appl. Neurobiol.*, **1**, 189-202; 1975).

Most of our knowledge of scrapie has come from studies of the disease in mice. The length of incubation is influenced by many factors such as the amount of agent and the genotype of mouse and, depending on these variables, it can be as short as 100 days or may exceed the lifespan of the host (Dickinson *et al.*, *Nature*, **256**, 732-733; 1975). Particularly impressive is the remarkable predictability of incubation period under defined conditions of infection. In most cases the incubation time can be measured with a standard error of only 1 or 2% of the mean suggesting an almost clockwork-like precision in the development of disease. Multiplication of the agent occupies much of the incubation period. When peripheral routes of infection are used the agent multiplies initially in extra-

Recent developments in scrapie

from Richard H. Kimberlin

neural tissues such as the spleen. Only later does agent become detectable in brain and the amount of agent increases slowly until clinically recognisable disease develops.

There is little doubt that the long incubation time is due to the slow rate of agent multiplication in brain. It is known that the dynamics of agent multiplication are under the control of a gene called *sinc* and there is increasing evidence that the slowness of the process is due to a host restriction on the number of replication sites. The most convincing evidence of this comes from the demonstration that the previous injection of a strain of agent that is "slow" in a given mouse genotype can completely block a "fast" agent injected later (Dickinson *et al.*, *Nature*, **253**, 556; 1975). The interference phenomenon is most simply explained in terms of the "slow" agent occupying replication sites which would otherwise be available to the "fast" agent.

To appreciate the significance of these findings it should be emphasised that no humoral or cell-mediated response to scrapie infection is apparent during the incubation period, that is, there is no evidence that "competition" between agents has an immunological basis. Similarly, prolonged depression of T or B lymphocyte functions has no effect on incubation period and there is substantial evidence that interferon plays no significant role in scrapie pathogenesis.

Despite the limited role of conventional host defence mechanisms there are clear indications of some kind of scrapie inactivation system in mice. Moreover, the importance of spleen (and probably other extraneural tissues) in the early multiplication of agent indicates the importance of some components of the lymphoreticular system. The question is—which cells are involved in these scrapie-inactivation and scrapie-multiplication processes?

Even though there is no evidence of an inflammatory reaction in scrapie brain there is a suggestion that an in-

flammatory response may be important because it is now known that injection of mice with prednisone acetate or with arachis oil (both of which suppress inflammation) can prolong the incubation period (Outram *et al.*, *Lancet*, **i**, 198-200; 1975). It is particularly interesting that these drug treatments need only be given for a very short period around the time of scrapie infection by a peripheral route, suggesting that they influence some of the cellular events involved in determining the "speed" of the scrapie "clock". These early interactions between scrapie agents and host cells will clearly be of great importance in future studies of scrapie pathogenesis.

The physicochemical nature of scrapie agents is another fundamental question that needs to be answered. This has been an exceptionally difficult problem to study for several reasons—no one has consistently identified virus-like particles in scrapie preparations using the electron microscope, so we don't really know what kind of agent to look for and immunological and tissue culture methods are not available for measuring amounts of agent. This can only be done by infectivity assays which in mice are very time consuming because of the long incubation period. Also, no-one has succeeded in obtaining purified preparations of scrapie agent which would make characterisation so much easier. Many standard virological techniques are therefore inapplicable to scrapie research and somewhat indirect methods have been used, mainly based on studying the effects of various physical and chemical treatments on scrapie infectivity (Hunter, *Prog. med. Virol.*, **18**, 289-306; 1974). These studies have revealed a number of important clues.

In general, scrapie agents are very stable to many treatments which would be expected to inactivate most conventional viruses. This stability may be related to the intimate association of infectivity with cell membranes. The smallest membrane fragments with infectivity have a diameter less than 50 nm. Important studies by Haig, Alper and others of the effects of ultraviolet and ionising radiation suggest, however, that the scrapie-specific portion of infective membrane fragments (the portion that codes for different strains of scrapie) may be much smaller. Despite past controversy, there is no good reason why the scrapie-specific portion should not be nucleic acid, in which case the ionising radia-

tion data suggests that it may have a molecular weight of 150,000 daltons (about the same size as viroid RNA). Therefore the simplest working hypothesis suggests that the infectious scrapie agent is made up of a small specific nucleic acid intimately associated with membranes of the host cell. Implicit in this model is the concept that scrapie agents do not exist as discrete virions which can be easily separated from cell components.

Against this background it is interesting to read the paper by Cho and Greig on page 685 of this issue of *Nature* in which the isolation of 14 nm virus-like particles from mouse brain infected with scrapie agent is described. The authors have been very careful to state that "it is not known whether these particles are indeed the scrapie agent" which is an honest admission that so far there is no evidence of whether these particles have scrapie activity. This is an important point because experience in cancer research has taught us to be wary of accepting an aetio-

logical role for viruses isolated from diseased tissues. The central nervous system is a well known repository of persistent and latent viruses which may or may not be involved in disease. It is quite conceivable that the particles isolated by Cho and Greig represent a virus which is present in brain and may be activated by scrapie infection but which plays no role in the disease.

The crucial questions to answer are do these particles *per se* have high specific scrapie activity and can they be isolated from other tissues such as spleen and from other hosts infected with other strains of agent?

If the answer to both these questions is yes then the findings of Cho and Greig will considerably alter our thinking on the nature of scrapie agents but, more importantly, they will provide purified particles that will make physicochemical characterisation so much easier. If the answer is no, then the authors have apparently purified a virus-like particle which may still be of interest because of its small size. □

More than meets the eye

by Miranda Robertson

The Brain Research Association Summer School, held in Cambridge on September 22-24, was followed by the Annual Meeting of the Institute of Biology, on the Physiology of Vision, in London on September 25 and 26.

THE dominant theme of both meetings was the interpretation of brain function in terms of the anatomy, physiology and psychophysics of its sensory systems. Patrick Wall, introducing a session on receptor systems at the BRA, remarked on a recent trend towards explaining perceptual phenomena purely on the basis of receptor mechanics, which he identifies as a resurgence of mechanism, traceable to Mountcastle at Johns Hopkins.

Photoreceptor interactions

The success of that approach at the sensory periphery was vividly illustrated by Trevor Lamb (Cambridge University), who explicitly related established psychophysical phenomena such as Weber's law and the Stiles-Crawford effect to the results of intracellular recordings from cones in the turtle retina (Baylor and Hodgkin, *J. Physiol., Lond.*, **242**, 729; 1974 and Baylor and Fettiplace, *ibid.*, **248**, 433; 1975). His concluding report was on electrical coupling between photoreceptors which at first sight seems

counter-adaptive since it degrades visual resolution. A prominent effect, and perhaps the function of such coupling is to reduce the intrinsic dark noise which apparently results from random opening and closing of the light-sensitive ionic channels in the photoreceptor membrane (Simon *et al.*, *Nature*, **256**, 661; 1975).

Lamb and his colleagues now have evidence in support of the notion that noise level in a single receptor depends on the extent of coupling to other cells. Very noisy cones turn out to have narrow receptive fields, whereas quieter ones respond to stimuli over much broader areas. Quantitative studies based on the degree of noise reduction are now under way to elucidate the pattern of coupling.

The question of receptor interactions arose again at the meeting of the Institute of Biology, where John Scholes (Kings College London) presented evidence for systematic (and probably antagonistic) interactions between red and green and green and blue cones in the retinae of rudd and goldfish, where the different photoreceptors can be distinguished morphologically. The same pattern of interaction is reiterated in the bipolar and ganglion cells fed by the photoreceptors; which makes it all the more fascinating that embryologically, the photoreceptor and its bipolar and ganglion cells all seem to have the same

origin in the lateral growth zone. That is the conclusion Scholes has drawn from labelling DNA in the growing fish retina, where the label appears in synchronised waves in the red, or the blue, or the green cones but is never seen to spread laterally in the receptor layer, and so must presumably be travelling vertically.

Spatial frequency channels

Lamb's talk was followed by a scintillating review from Fergus Campbell (Cambridge University) on the psychophysical evidence for spatial frequency channels in human vision, which are not explicable at the receptor level. Horace Barlow, from the chair, raised the question of how to explain, at any level, how the Fourier analysis which Campbell has suggested as the basis for the frequency response can be combined with the retention of precise positional information. Campbell's response was to concede that the Fourier analysis must be done piecewise, so that much of the information in the frequency channels is positional and only some of it is broken down into spatial frequencies. As the physiological substrate for this dichotomy, however, he suggested that spatial frequencies might be coded in the large, fast-adapting Y fibres of the optic nerve and position in the slow X fibres. That remark elicited a fascinating case history from Geoffrey Arden, who has been using new television techniques for recording visual evoked potentials from multiple sclerosis patients. They can be shown initially to suffer the selective loss of their X fibres (*The Evoked Potential*, edit. by Desmedt, J., Oxford University Press, in the press), and they complain, not of the loss of detailed vision, but that they cannot tell the position of an object if it is moving.

Hard wiring in the cortex?

The mechanistic approach remarked by Wall at the BRA meeting is at its most impressive in the visual cortex, where, as Wall pointed out, the beginnings of abstraction are to be seen in the existence of cells which respond selectively to lines of a given orientation. A. J. Movshon (Cambridge University) briefly sketched the history of the controversy which still exists over the part played by postnatal experience in determining orientation selectivity, concluding with some arresting recent evidence in favour of it.

Movshon has followed up the experiments of Pettigrew and Blakemore on the reversal of cortical ocular dominance as a result of suturing first one eye and then the other eye of kittens during the sensitive period. By micro-electrode recordings across the cortex

of a series of these animals at different times before and after suture, he was able to follow the expansion and contraction of the columns as first one eye and then the other took control.

That process is not controversial, and has actually been photographed by D. Hubel (Harvard University) who showed some amazing slides of coronal sections of monkey visual cortex stained by the injection of labelled amino acid into one eye. The zebra stripes formed across the cortex by the ocular dominance columns were caught in the act of reciprocal contraction and expansion as a result of suture reversal, leaving no room for doubt about what was going on.

The disagreement arises over orientation selectivity, which Movshon and his associates claim is influenced by visual experience and Hubel and Wiesel claim is innately specified. Movshon's evidence comes from recordings of a few cells in reverse sutured kittens that could be stimulated from either eye. Such binocular cells in normal cats invariably have the same orientation selectivity for stimuli from right and left eyes. In the reverse sutured kittens, however, Movshon found a few binocular cells, presumably in the process of changing their ocular allegiance, which seemed at the same time to be changing their orientation preference. These cells were selective for different orientations when stimulated through the different eyes.

While that would seem to be compelling evidence for plasticity under the positive influence of experience in the cat, Hubel has begun to test the effects of stripe-rearing (in which visual experience is limited to bars of a single orientation) in monkeys and (in the one satisfactory specimen so far) has found no effect on the columnar pattern of orientation selectivity of cortical cells. Challenged by Movshon, he could offer no explanation for the discrepancies; nor could Movshon explain recent failures to replicate stripe-rearing results in cats.

Perceptual problems

Another embarrassment for visual neurophysiologists emerges from psychophysical experiments on stereopsis described by J. P. Frisby (University of Sheffield). According to the neurophysiology, stereopsis is mediated by binocular, orientation-selective cells which have a marked maximum response where the stimuli in either eye have a specific horizontal disparity on the retina. Frisby has tested depth perception in man (two men, himself and one other) using an adaptation of the Julesz random dot stereogram made with lines instead of dots. He has found that the perceived depth is a function of the length of the lines

Assessing prospects for Spacelab

from John Gribbin

A FEW months ago a series of experiments were flown on board NASA's CV-990 'flying laboratory' to simulate an orbital mission by the European Spacelab which is to be flown on the Space Shuttle during the 1980s. Some of the first scientific results from this ASSESS mission are presented in a series of papers commencing on page 649 of this issue, but the other lessons learned are also of great interest.

The joint ASSESS mission illustrated that a low-cost programme with a low level of preparatory requirements, testing and documentation can operate successfully under the proper management. Not only was the ASSESS mission itself fairly cheap (by space agency standards); it provided every indication that low-cost projects can work well when the real Spacelab gets off the ground, which must be encouraging news for many of the research groups working within tight budgets to try to take advantage of the opportunities which will be provided by the next generation of manned spacecraft.

And such aircraft simulations may have a significant role to play in developing instrumentation for the next generation of unmanned spacecraft as well. The ASSESS flights included an experiment from the Laboratory for Atmospheric and Space Physics (LASP) of the University of Colorado, a scanning two-channel spectrometer

which covered the wavelength range 270–650 nm and was a prototype of the ultraviolet spectrometer which LASP expects to fly on the forthcoming Pioneer Venus orbiter spacecraft. The mission provided a far better opportunity to sort out bugs in the system than any ground based operation, as well as producing valuable observations of Venus.

But ASSESS proved that there will be real problems in the practical operation of a flying laboratory. Human engineering of instrument control is necessary, as is a near real time high quality visual display of recorded data to confirm to the operator that successful measurements are being made. One clear result emerged from the human side of ASSESS. The best results come from operators who are trained scientists, understand the equipment and can use a soldering iron effectively on the spot to overcome the troubles they have diagnosed. There will, it seems, be no retreated test pilots on the Spacelab, only a professional astronaut-pilot and the professional scientist-operators.

A second mission is now planned. NASA has indicated that the CV-990 will be available in October/November 1976, which would allow time for the data collected to be usefully incorporated into the final stages of the development of Spacelab.

used, a result which defies explanation within the current physiological paradigm.

No satisfactory physiological paradigm has in any case yet been proposed for perceptual phenomena which depend on activity beyond the primary visual cortex, a point implicit in the title to which Stuart Sutherland (University of Sussex) addressed himself: "There's more to vision than meets the eye." Sutherland believes in the use of computer programs as a testable way of representing how feats such as human visual perception might be achieved, and the first lesson to be learned from such programs is the enormous amount of knowledge and past experience people bring to bear on seeing things (consider the difference between a layman and a trained histologist, looking at a microscope slide). The lesson from the current lumbering knowledge-based computer vision programs however seems to be that brains have some way of leaping to global conclusions while computers are still creeping along line by line. □

Protoplasts at Nottingham

from E. C. Cocking

The 4th International Meeting on Yeast and Other Protoplasts was held at Nottingham, UK on September 8–12. The proceedings will be published by Academic Press.

THIS was one of the rare occasions when workers on bacterial, fungal and higher plant protoplasts came together to exchange viewpoints. As a result it was possible to compare the properties of protoplasts from both eukaryotic and prokaryotic systems; and for many of the participants this was the major attraction.

Enzymatic proceedings for the degradation of the cell wall, resulting in protoplast release, are now largely standard procedures; and, although the enzymes used for isolation differ in the various systems, the influence of the exact nature of the cell wall was

central in all the discussions of protoplast isolation. The bacterial membrane was shown by R. E. Marquis and T. R. Corner (Rochester University, New York) to be a remarkably extensible structure, while the membrane surrounding fungal protoplasts attracted the attention of D. Kerridge *et al.* (Cambridge University) to ascertain whether difference in chemical composition of plasma membranes, from different growth stages in the growth cycle, could be correlated with differing sensitivities to polyenes.

The activity of the plasma membrane in the synthesis of a new cell wall around isolated protoplasts was described in detail for all three protoplast systems. The induced, heritable, condition of wall-less-ness in bacteria was shown not to be due to alteration in the DNA. No comparable stable L forms have been found to arise from fungal protoplasts, or from higher plant protoplasts. But Y. Meyer *et al.* (Heidelberg University) described special conditions under which higher plant protoplasts regenerate a non-rigid, polysaccharide containing, "pseudo-wall". The photosynthetic activity of plant leaf protoplasts was very lucidly described by G. E. Edwards *et al.* (University of Wisconsin). It is readily possible to compare the photosynthetic activities of protoplasts isolated from C3 and C4 plants. Indeed, such protoplasts are more useful than isolated chloroplasts for certain photosynthetic studies, since enzymes of the cytoplasm, peroxisomes and mitochondria may be involved in carbon metabolism during photosynthesis.

As might be expected, great interest centred on the activity of plasma membranes in the fusion of isolated protoplasts. Fusion of bacterial protoplasts is still neglected; work on the polyethylene glycol induced fusion of fungal protoplasts, is however, actively continuing, and L. Ferenczy *et al.* (Szeged University) and J. Anné (Louvain University) and J. F. Peberdy (University of Nottingham), were able to show how fusion was expressed by heterokaryon formation between nutritionally complementing strains. Until recently, the only means of hybridisation in plants was by sexual crossing; E. C. Cocking (University of Nottingham) surveyed the ever increasing momentum of work on an "alternative to sex" which involves the induced fusion of somatic plant protoplasts, and, following adequate selection and culture, the regeneration of hybrid plants; the importance of combining this somatic hybridisation with the sexual genetics of higher plants was highlighted. Following the report earlier this year of the induced fusion of yeast protoplasts with hen erythrocytes, con-

siderable interest was aroused by the report of M. R. Davey and J. B. Power (University of Nottingham) of ultrastructural studies which showed that the induced fusion of the yeast plasma membrane with that of higher plant protoplasts takes place within uptake vesicles, following treatment of mixtures of these protoplasts with polyethylene glycol.

F. E. Young *et al.* (Rochester University) showed how the bacterial surface plays an extremely important role in the uptake of DNA and adsorption of viruses. In the eukaryotic fungal and higher plant systems comparable studies are at a much earlier stage of development, and there are many technical difficulties still to be resolved. It was encouraging, therefore, that D. Hess (Hohenheim University) was able to show that there is a considerable enhancement of DNA uptake when the DNA is fed as a calcium phosphate complex. Also encouraging were reports from several laboratories that polyethylene glycol may be a useful agent in attempts to establish endosymbiotic associations between higher plant cells and microorganisms *in vitro*. Those more physiologically orientated workers, interested in cell fractionation, noted with interest the report of T. Boller *et al.* (ETH, Zurich) of the use of the polybase, DEAE-dextran, for the isolation of vacuoles by the lysis of yeast protoplasts.

There is no doubt that this meeting fulfilled a long felt need for greater contact between those working on prokaryotic and eukaryotic systems. When the next International Meeting is held in Szeged, Hungary, in 1978 it is intended to extend further this induced fusion of protoplast disciplines.

Problems in the derivation of mRNA

from a Correspondent

The third Arolla workshop on the formation of messenger RNA in eukaryotic cells was organised by Klaus Scherrer at Arolla, Switzerland from September 2-6.

As on the two previous occasions, the main focus was on mRNA synthesis, but related topics were included. The subject matter was therefore gene expression in the round.

The meeting opened with reviews of chromatin structure and the characterisation of nonhistone proteins. Despite universal agreement that chromatin has a beaded structure, containing discrete protein clusters, there is still considerable doubt both as to the organisation of the clusters and on the relationship

between the histones and DNA. Histone H1, which shows microheterogeneity and a varying mass ratio to DNA, is not integrally involved in the structure of the beads, which appear to be octamers containing two molecules each of histones H2A, H2B, H3 and H4. The authenticity of reconstituted preparations of chromatin remains in some doubt, since they do not generate the array of fragments containing multiples of 200 base pairs of DNA which is characteristic of mildly digested native chromatin. Whether reconstituted preparations can serve as accurate templates for transcription *in vitro* remains controversial.

The nonhistone proteins comprise a dauntingly large number of species which vary in molecular weight from less than 10,000 to more than 200,000 daltons. There was no indication of any hope that the problem of defining these proteins is nearer solution. The observation that 20% of the proteins bound to DNA in low salt are removed below 0.2 M and 20% above 3 M may help to explain conflicting views on the metabolic stability of nonhistone proteins. Progress has been made in identifying increasing numbers of prominent nonhistone protein components, including DNA ligase, DNA polymerase, histone kinase, histone methylase, actin, myosin, tropomyosin and α and β tubulins. But while this is valuable information in its own right, we cannot seriously hope to identify the putative regulatory proteins by a process of elimination.

Discussion of satellite DNAs *per se* gave the impression that progress in this area is now slow, perhaps inevitably, since the structure of the satellites is by now understood quite well while their possible functions are much less amenable to investigation. But a satellite-like structure of small scale tandem repetition has been observed in the nontranscribed spacer of ribosomal DNA, which displays microvariation in length. One possible function for the spacer in this system may be to help maintain the homogeneity of transcribed DNA sequences by increasing the frequency of unequal crossing over. Another tandemly repeated DNA sequence on which rapid progress has been made is, of course, the histone DNA. There appears to be a basic repeat unit which contains the sequences for all five histones, separated by short spacer regions. Whether a single precursor for all the histones is transcribed is not known. Progress with both rDNA and histone DNA has clearly received a tremendous boost from the successful cloning of the DNA by means of bacterial plasmids.

Bridging the gap between DNA organisation and chromatin structure on the one hand and on the other the

characterisation of the primary transcription product, there was a summary of recent electron micrographic observations on the active interphase chromatin of *Drosophila* nurse cells. This work has generated the first description, in these terms, of nonribosomal transcription units. In most cases, when the lengths of RNP fibres were plotted against distance along the DNP axis, a clear correlation extended over lengths averaging 3.2 μm . One appealing interpretation of these results is that each observed transcription unit is equivalent to a single cistron as defined by genetic analysis, with an apparent average length of 6 μm . These results contrast with biochemical observations of much smaller nuclear RNA molecules, a paradox whose resolution may help define the derivation of mRNA.

The existence of a giant nuclear precursor of mRNA remains controversial. Work on duck erythroblasts, mouse Friend cells, hen oviducts and L cells was reported; but there is no clear consensus on the characteristics of any precursors. The failure of ten years of effort to solve this problem suggests strongly that new techniques may be needed, perhaps for example the use of plasmid clones of appropriate sequences. Work in one system stands out, however; it was reported that only 20–25% of the total 75S product of Balbiani ring 2 of *Chironomus* salivary glands ever reaches the nucleoplasm and only 5% enters the cytoplasm. If this does not merely reflect the sickness of explanted glands, it would suggest a powerful processing component in gene expression.

In a session devoted to the ends of eukaryotic mRNA molecules, some possible functions emerged for 3' poly(A) sequences and 5' methylated caps. If the length of poly(A) on globin mRNA is reduced to less than 40 residues, its decay after injection into oocytes is accelerated. Decay is very rapid when the length is less than 15 residues. The validity of this observation is supported by the fact that during the later stages of reticulocyte maturation the length of the poly(A) of the mRNA that can be recovered remains a constant 40–50 residues, despite a net fall in the amount of mRNA per cell. Viral mRNAs which normally are capped require methylation of the 5' terminal guanine for translation in the wheat germ system. In this system methylation is required for formation of the initiation complex, and from this a 35 nucleotide 5' terminal fragment can be isolated following treatment with ribonuclease. Picorna viruses, which normally are not capped, are not translated in this system and are translated without methylation in others.

Much progress has been made in sequencing studies. Partial sequences of several purified poly(A)-containing mRNAs now are available; in several cases it is clear that there is a significant length of nontranslated RNA at each end, longer at the 3' end. Sea urchin histone messengers also contain a nontranslated region. Rabbit α and β globin mRNAs and a mouse immunoglobulin L chain mRNA contain a 3' terminal sequence which suggests the existence of two hairpin loops which may or may not be stable *in vivo*. If the loops are present, these three messengers contain the same short sequence along the spine of the molecule. Hen ovalbumin mRNA does not contain this sequence. But the tetranucleotide adjacent to the poly(A) moiety is the same in all four messengers; this might be part of signals concerned, for example, with termination of transcription or polyadenylation.

The inherent tendency of hnRNA to form aggregates makes doubtful many of the conclusions based on fractionation by means of affinity columns, since these are often operated under conditions likely to promote aggregation, and so molecules with no affinity might be aggregated with those that have and thus be retained. A promising approach to the structure of hnRNA appears to be the analysis of the smaller sequences obtained by fingerprinting T_1 digests of total hnRNA and analysis of the small (3%) residue which is resistant to ribonuclease A. Sequence analysis of the ribonuclease-resistant hairpins suggests that many may have the same or a similar sequence. This family of sequences has some 10^5 members, each perhaps 150 bases in length, and should correspond to something less than 1% of the mammalian genome. A fingerprint pattern containing some characteristics of the ribonuclease-resistant RNA is obtained from both the hnRNA and mRNA which hybridise with DNA at very low C_{ot} values. There were indications of a relationship between the RNase-resistant hnRNA hairpins and the S1-resistant foldback loops of DNA. The distribution in CsCl gradients of the DNA sequences complementary to hnRNA hairpins is different from the bulk DNA distribution, which would be consistent with some clustering of these sequences in DNA.

Since no enzymes which cleave RNA are known in eukaryotes attention was focused on those found in bacteria, where it seems that several different classes of recognition process are involved. Five hexanucleotide sites on RNA cleaved by RNase P are all different. And mutational changes in different parts of the tRNA precursor affect its processing; some of these are near the cleavage site, some are in the

complementary part of the molecule, and some are quite remote. On the other hand, the known 5' termini of T7 RNA fragments all have very similar sequences. One general point of obvious importance is the observation that hairpin loops are invariably implicated in bacterial processing reactions. A rather striking observation is that RNase D (=Q=III) can cleave 45S ribosomal precursor to yield a molecule sedimenting at 28S; however, characterisation of the product clearly is necessary before this can be interpreted. A feature of this enzyme is that it preferentially cleaves at specific sites in low salt, but preferentially solubilises double-stranded RNA in high salt; this reinforces the view that the products of its action must be carefully characterised. $\square$

Dale centennial celebrations at Cambridge

from L. L. Iversen

The Sir Henry Dale Centennial Symposium—Post-synaptic Actions of Neurotransmitters was held in Cambridge on September 17–19.

IN the days when Sir Henry Dale made his pioneering contributions to the understanding of the actions of histamine, adrenaline and acetylcholine (ACh) on various tissues including those of the nervous system, he deliberately avoided the use of the term "receptor", then used in a purely operational sense. Such reticence is no longer necessary, as was amply demonstrated by the up-to-date reviews of drug-receptor interactions presented at the symposium.

The highest development of such studies was described by J. P. Changeux (Pasteur Institute, Paris), reviewing the work of his group on the purification and characterisation of the nicotinic cholinergic receptor protein from excitable membranes of the electric organs of fish. Changeux described a model for the action of the ACh receptor which postulates the existence of the receptor in three possible states: resting, activated and—after prolonged exposure to ACh or a related agonist—a desensitised condition. Desensitisation can be demonstrated *in vitro* using membrane fragments exposed to carbachol, and results in binding of ACh and other agonists with unusually high affinity, but without transduction into a change in membrane sodium permeability, as in the normal transition from resting to activated states.

Remarkable new refinements in the neurophysiological study of ACh at the

neuromuscular junction were reported by Sir Bernard Katz (University College, London) and S. Kuffler (Harvard Medical School). Katz described how statistical analysis of the increased muscle membrane noise generated on exposure to ACh has shown the precise characteristics of the changes in membrane conductance elicited by ACh in individual membrane ionic gates or channels. Kuffler described work by his group on snake muscle, in which it is possible to apply measured amounts of ACh directly to the endplate areas of muscle membrane from which the nerve terminal has been removed by microdissection. They have established that less than 10,000 molecules of ACh are needed to mimic the effects of one quantum of transmitter normally released by the nerve terminals (several hundred such packages are released by each nerve impulse).

A. S. V. Burgen (National Institute for Medical Research, London) described rapid progress in characterisation of muscarinic cholinergic receptors. The binding characteristics of labelled antagonist drugs to muscarinic receptors in smooth muscle or brain correspond well to those predicted from previous pharmacological experiments, but those of agonists seem more complex. Graphs of agonist binding followed non-linear Hill plots, suggestive of complex cooperative interactions with the muscarinic receptor sites, or, more probably, of the existence of more than one category of agonist binding site. The latter possibility is in accord with the earlier findings of Spero and Burgen, that agonist effects on ion fluxes and contraction in smooth muscle are not always parallel. When a covalent alkylating reagent for muscarinic sites is used, N-propylbenzylcholine mustard, the muscarinic receptor molecule in smooth muscle can be identified as a polypeptide with a molecular weight of 85,000.

P. Greengard (Yale University) reviewed evidence that the post-synaptic actions of ACh at muscarinic receptors and of other transmitters, notably noradrenaline and dopamine in the brain, may be mediated by changes in the concentrations of cyclic AMP or cyclic GMP in the post-synaptic cells as a result of activation of a transmitter-sensitive adenylate or guanylate cyclase in the synaptic membrane. Recent work supports the hypothesis that cyclic nucleotides may produce synaptic potentials by activation of a protein kinase that in turn alters the level of phosphorylation of proteins in the synaptic membrane and consequently the ion permeability characteristics of the cell. In synaptic membranes from brain, cyclic AMP activated the phosphorylation of a few specific membrane proteins, some of which have

been purified. In a simpler system, the turkey red blood cell, which responds to catecholamines by changes in ion permeability through a cyclic AMP mediated β -adrenoceptor mechanism, it was possible to show a good correlation between the extent of increased phosphorylation of a specific membrane protein and the associated changes in intracellular cyclic AMP and ion fluxes caused by exposure to catecholamines.

Experiments on dopamine receptors in mammalian brain, using radioactively-labelled dopamine or the antagonist drug haloperidol as ligands, were described by S. Snyder (Johns Hopkins University). The data on the direct interaction of agonists and antagonists with CNS dopamine receptors complemented those of Greengard and others from studies of drug interactions with the dopamine-sensitive adenylate cyclase in mammalian brain. As with several other transmitter and drug receptors, there was evidence that dopamine receptors could exist in two stable configurations, one with a high affinity for agonists, and the other with lower affinity for agonists but a high affinity for antagonists.

D. Jenkinson and J. Black (University College, London) discussed the dual receptor systems that mediate adrenaline and histamine responses in the body, the α - and β -adrenoceptors, and H1 and H2 histamine receptors. The use of radioactively labelled β -adrenoceptor antagonists, such as alprenolol, allows the ligand binding approach to be applied here also. The possible interrelations between the two types of adrenoceptor remain tantalising but obscure; Jenkinson's own group has recently shown that β -adrenoceptor agonists could elicit changes in ion fluxes in isolated tissues only after a previous exposure to α -adrenoceptor stimulation.

G. Aghajanian (Yale University) described the work of his group on the neurophysiological characterisation of receptors for 5-hydroxytryptamine (5-HT) in mammalian brain. The actions of 5-HT on receptors located on the surface of the 5-HT neurones themselves are very potently mimicked by *d*-LSD and related hallucinogens, which thus cause a powerful inhibition of the firing of 5-HT neurones in the brain.

Surface bound antibodies on B lymphocytes and on mast cells have many similarities to the receptor macromolecules of excitable tissues, and M. Raff (University College, London) reviewed the work of his group on the unexpected lateral mobility of such ligand-binding proteins in the cell membrane. Recent fine structural studies of histamine release from mast cells throw new light on the mechanism of exocytosis in which cell membrane protein seems to be conserved by migration from

regions of contact between secretory vesicles and plasma membrane.

Finally J. Axelrod (National Institute of Mental Health) reviewed work by his group on the longer term changes that are initiated by transmitter molecules impinging on post-synaptic tissues. In adrenergic neurones and in the pineal gland the extent of synaptic stimulation controls the rate of synthesis or degradation of key enzymes in the biosynthesis of catecholamines or the pineal substance melatonin. The pineal gland in particular offers an elegant model system for studies of the rapid and complex changes that can occur in protein synthesis, enzyme activity and the sensitivity of the β -adrenoceptors to stimulation by catecholamines. Changes in receptor sensitivity leading to supersensitivity (with decreased stimulation) or subsensitivity (on prolonged stimulation) can occur in this tissue within a few hours and are associated with parallel alterations in the number of β -adrenoceptors detectable by radioactive alprenolol binding studies. □

Crystallographers in Amsterdam

from Olga Kennard

The 10th International Congress of Crystallography was held in Amsterdam on August 7-15.

AMSTERDAM in the heat wave, with flags flying, sunlight glittering on the canals and 1,500 crystallographers gathered for their 10th Congress—29 years after the foundation of the International Union of Crystallography and some 63 years since the beginning of X-ray crystallography. A young discipline—and even the disciples have hardly aged. The pace of the Congress was set by Paul Ewald, now 87, who not only gave a memorable discourse, but who, by his penetrating questions, made many a younger contributor re-examine his basic assumptions.

Crystallography is still full of excitement and experimentation. Experimentation even in scientific communication, which at Amsterdam meant great emphasis on poster sessions held in specially constructed booths complete with tables, chairs and blackboards—veritable psychiatric clinics! Altogether some 600 contributors opted for posters compared with 300 short oral papers. The sense of "discussions in corridors" prevailed in the booths as shown by the continuous and spirited arguments which otherwise so rarely develop in lecture rooms after short presentations.

Poster sessions were complemented

by invited lectures. Some such as Phillip Coppens's (State University of New York, Buffalo) review of the accurate determination of electron densities, drew together and made coherent sense from a scatter of individual exhibits and gave the necessary background and critical evaluation for the non-specialist. More of these link-lectures would ensure even greater effectiveness of the poster sessions at future Congresses.

The eight general lectures were all delivered with great style and high information content. Caroline MacGillavry's discourse on "Order and Beauty" continued the old crystallographic tradition of linking the two cultures. A. I. Kitaigorodski's (Institute of Elemento-Organic Compounds, Moscow) talk on "Interaction in molecular and non-molecular crystals and lattice dynamics" should perhaps be mentioned for its simplicity of approach, which set a good example, especially to the younger generation of computer-bred crystallographers juggling with too many variables. His definition of scientific theory: first rate if it can predict, second rate if it can forbid and third rate if it can explain "post factum", became a useful yardstick for the Congress.

There were 27 main topics covering all aspects of crystallography from perfect crystals to almost total disorder. Macromolecules, both globular and fibrous, were well represented, with an impressive exhibit of models, and considerable emphasis on the functional and evolutionary aspects. There seems to be an imminent breakthrough in virus structures and a particularly exciting account was given by John Champness (MRC Laboratory of Molecular Biology, Cambridge) on the structure of the disk of tobacco mosaic virus protein to 5 Å resolution.

It would be tempting for this rapporteur to discuss small biological molecules, for example the first phospholipid and membrane component structures (R. Mason, University of Sussex; S. Abrahamsson, University of Göteborg; and M. Sundaralingam, University of Wisconsin, Madison) but invidious if some fascinating compounds in the six other structural sections are neglected. The general impression was that crystallographers have now overcome their sense of guilt at the comparative ease with which structures can be solved and are turning their attention to a more fundamental interpretation of the results in terms of physicochemical properties, function or mode of action.

Not all structures yield to current techniques and one of the highlights of the meeting was the session on direct mathematical methods. H. Hauptman (Medical Foundation of Buffalo) set the

theme with a general talk which included the new quartet invariants—a true breakthrough! P. Main (York University) discussed the use of known chemical information and his matrix approach looks very promising.

B. Busetta (University of Bordeaux) applying these methods, reported the solution of perhaps the largest "small" structures to date with 116 and 126 light atoms in the asymmetric units of difficult, non-centric space groups. Rumour has it, however, that the Karles (Naval Research Laboratories, Washington) still keep the record, with a modification of valinomycin (156 atoms in space group P1). These new developments offer real promise to view, without recourse to heavy atoms, the structure of compounds perhaps as large as 3,000–5,000 daltons, at atomic resolutions.

An interesting feature of the exhibits was a structure-solving clinic using a minicomputer (G. Sheldrick, Cambridge University; E. Oeser, Technische Hochschule, Darmstadt). Sixteen apparently insoluble structures were submitted and six were actually solved and refined during the Congress. Need sponsors have any better proof of the value of international meetings!

A final word about the small community of Dutch crystallographers. They organised the Congress with imagination (the brass band of the Amsterdam Police instead of welcoming speeches!) and a friendly efficiency which ensured the highest possible satisfaction quotient—both scientific and social—for all participants. □

Vaccination against fertility

from a Correspondent

The Third International Symposium on the Immunology of Reproduction was held in Varna, Bulgaria on September 21–25. It was supported by the Union of Scientific Workers in Bulgaria, the G. Dimitrov Agricultural Academy, the Bulgarian Academy of Sciences, the Ministry of Health in Bulgaria and the World Health Organisation. The proceedings will be published by the Bulgarian Academy of Sciences.

THE proceedings in Varna revealed good prospects for an anti-fertility vaccine, potentially invaluable in the battle against world overpopulation. Of particular interest were the initial findings of G. P. Talwar (All India Institute of Medical Sciences, New Delhi) who injected a few women with the specific portion (the beta subunit) of human chorionic gonadotropin

conjugated with tetanus toxoid. The antibody titre to the gonadotropin rose to high levels which were maintained for more than a year, and the antibody was shown to inhibit the biological activity of the gonadotropin, which plays an essential role in pregnancy. In one case, a booster inoculation was given when the antibody titre fell and this was followed by a return to former levels. Tests on a wide range of bodily functions provided no evidence of any undesirable side-effects of the treatment. The women had previously requested surgical sterilisation and menstrual cycles continued throughout the period of observation. Similar studies to Talwar's are being initiated in several institutions in Europe and the Americas, so that thorough assessment of the safety of the method should be accomplished in the near future. Large-scale clinical trials will follow if indications are favourable. In spite of Talwar's apparent breakthrough, however, detailed studies are continuing in various laboratories on the synthesis and biological activity of amino acid chains representing parts of the non-homologous region of the beta subunit which could prove highly specific antigens. The possibility of immunisation with other specific placental proteins is also being pursued actively, chiefly with components identified as SP1 and PP5.

Probably the largest single topic of the symposium had to do with the antigenic structure of spermatozoa and their immunological potential in both the male and the female. In the human male the concern was primarily with infertility associated with anti-sperm antibodies in the circulation, and with the risk of autoimmune damage in vasectomised men. The impressively extensive use of vasectomy in India was described by S. S. Rao (Institute for Research in Reproduction, Bombay) who noted that many subjects were found to have high titres of circulating antibody apparently without serious consequence. The prospects of developing a contraceptive vaccine for males as an alternative to vasectomy has been improved by the use of adjuvants with less drastic local effect than Freund's adjuvant. With the female the emphasis is on the control of fertility by immunisation with spermatozoa or specific sperm antigens, such as hyaluronidase, acrosin, LDH-X and antigen T. Progress in this field was usefully reviewed by W. R. Jones (Flinders University of South Australia, Adelaide). Antibodies against these antigens do not cross-react significantly with somatic tissue antigens, although human acrosin is very similar in amino acid composition to pancreatic trypsin. Some depression of fertility is achieved by active immunisation but the results are not yet

satisfactory. Curiously enough the reduction in fertility produced by anti-LDH-X antibodies is owing mainly to embryonic death during cleavage and implantation. The fact that sperm-agglutinating but non-immobilising sera could prevent sperm penetration through cervical mucus in an *in vitro* test was reported by B. Boettcher (University of Newcastle, New South Wales). S. Shulman (New York Medical College) noted the occurrence in cervical mucus of sperm-immobilising antibodies active in the absence of complement. S. Isojima (Hyogo Medical College, Nishinomiya) described an improved microtechnique for detecting sperm-immobilising antibodies in cervical mucus. The risks involved in the use of placental hormones and sperm antigens were considered critically by K. S. K. Tung (University of New Mexico, Albuquerque).

The foetus as a homograft received its share of attention, the feeling clearly being that the reasons for the lack of foetal rejection are still poorly understood. But further support was provided for the idea that genetic dissimilarity favoured implantation. □

Charting the cell cycle

from M. Vicente

The Third European Workshop on the Cell Cycle sponsored by the European Cell Biology Organisation was held in Edinburgh on September 23-25.

SIMPLE systems still seem to be leading the race for the understanding of the events constituting the cell cycle. Although the majority of the papers dealt with eukaryotic systems, the most interesting results were reported from studies of prokaryotes or relatively simple eukaryotes such as yeast and the slime mould *Physarum polycephalum*. These organisms share the property of being either single cells or plasmodia in which the life history of a growing individual is not heavily disturbed or dependent on the presence of other individuals or their metabolic products. Moreover all of them are suitable for biochemical and genetical studies.

M. Masters (University of Edinburgh) has produced strains of *Escherichia coli* in which a duplicate copy of the chromosomal origin of replication is carried on a plasmid. Such strains have a significantly reduced number of chromosomes per cell mass and also divide at a proportionally larger size. These properties are consistent with the idea that there is a fixed amount of some substance required for the initiation of DNA replication at the chromo-

some origin and that the presence of extra copies of this origin results in competition between the chromosome and the plasmid for initiation at each mass doubling.

Three communications by P. Thuriaux *et al.*, P. Fantes and P. Nurse (University of Edinburgh), gave a new view of the cell cycle in the fission yeast *Schizosaccharomyces pombe* obtained from the study of mutants in which progression through the cycle is blocked or altered at different stages. There is a close similarity between the cell cycle in *Saccharomyces cerevisiae* and that in *S. pombe* as described by Thuriaux *et al.* Both cycles are in turn very similar to the *E. coli* cycle summarised at the meeting by W. D. Donachie (University of Edinburgh). The cycles in the three species have two independent pathways, one involving chromosome replication, both of which are required for the normal progression of the cell towards division. There is also evidence suggesting that the pathways comprise several sequentially dependent reactions.

Homeostatic control of cell size in *S. pombe* was observed by P. Fantes studying a thermosensitive mutant blocked at a stage before nuclear division at the restrictive temperature. The treatment does not seem to disturb cell growth in other ways and allows cell elongation to proceed. In these circumstances a negative correlation between the length of a cell at division and the duration of the following cycle is observed once the cells are shifted back to the permissive temperature. Some of the processes leading to division are more dependent on time than on size, however, as no matter how long the cells become before the temperature release they always require a minimum time before they can divide.

That initiation of DNA synthesis can be controlled by size while nuclear division is controlled by time was suggested by Nurse when interpreting his observations in a *S. pombe* mutant in which mass at division is half the wild type value and DNA synthesis is displaced to a later stage of the cycle while timing of nuclear division remains unchanged. The simultaneous functioning of timing and size controls is one more aspect in which the cell cycle in *S. pombe* is analogous to that of *E. coli*.

Replicating DNA fibres of *S. cerevisiae* have been made visible by autoradiography, after pulses of radioactive uracil, by D. H. Williamson and T. D. Petes (National Institute for Medical Research, London). They find that DNA replication proceeds bidirectionally in most of the replicons starting from several replication origins in each fibre, as in other eukaryotes. Therefore most of the replicons seem to initiate synthesis very early in the S period as

replication becomes resistant to inhibition of protein synthesis once the S period has started.

A quite different replication pattern is present in *Physarum polycephalum* in which several protein-dependent initiation events are detected by cycloheximide inhibition. Studying replication of DNA during the step that takes place 30 min after mitosis, F. Haugli (University of Tromsø) has isolated, after short pulses of ³H-thymidine, 7S DNA fragments very similar to the *E. coli* Okazaki pieces. These fragments are gradually chased into longer intermediates by a process that closely resembles the joining by ligase in *E. coli*.

Both R. Braun and V. M. Vogt (University of Berne) and H. W. Sauer *et al.* (University of Konstanz) suggested that the DNA that codes for ribosomal RNA in *Physarum* is contained in a structure that resembles morphologically a bacterial plasmid. In addition Braun and Vogt have found that replication of this rDNA occurs at random during G2, a mode of replication that is consistent with that found in plasmids.

Reports on *Physarum* presented at the workshop show that studies on this organism are in a state comparable with that of *E. coli* in the early 1960s—that can be taken optimistically or pessimistically depending on how one views it. □



A hundred years ago

SIXTH REPORT OF THE SCIENCE COMMISSION

THREE times within the last twelve years a Royal Commission has reported on the science teaching of our higher schools. In 1864 the Public Schools Commission announced that from the largest and most famous schools of all it was practically excluded. In 1868 the Endowed Schools Commission declared that the majority of school teachers had accepted it as part of their school work. The Science Commissioners of 1875, in their Sixth Report, on Science Teaching in Schools, testing this statement by inquiry, reports that of 128 endowed schools examined by them not one-half has even attempted to introduce it, while of these only 13 possess a laboratory, and only 10 give to the subject as much as four hours a week. And this statement is curiously illustrated by the statistics of the recent Oxford and Cambridge School Examination, which show that out of 461 candidates for certificates from 40 first-class schools, while 438 boys took up Latin, 433 Greek, 455 Elementary Mathematics, 305 History; only 21 took up Mechanics, 28 Chemistry, 6 Botany, 15 Physical Geography. From *Nature*, 12, 549, October 28, 1875.

articles

Simulating experiments for Spacelab

In June this year a number of flights were made in a NASA CV990 aircraft to evaluate designs for experiments to be carried aboard Spacelab from 1980 onwards. Here five of the groups involved describe what they achieved. NASA and the European Space Agency recently decided to carry out another similar mission next year.

Measurements of ozone and minor atmospheric constituents

THE instrument flown was an absolute spectrometric radiometer for the spectral range $3\text{--}40\text{ cm}^{-1}$. Such an instrument on a space platform would be used to measure the cosmic background spectrum and to monitor atmospheric constituents. At aircraft altitudes the atmospheric emission prevents the first, and the scientific objectives set for the flights were therefore to explore the spectral assignments and concentrations of ozone and minor constituents of the atmosphere and in so doing to test developments of technique. Previous experiments had shown the need for improved techniques that would give a resolution of at least 0.01 cm^{-1} unapodised so that overlapping emission lines from different constituents are resolved, that would allow an interferogram to be recorded in no more than a few minutes because significant changes can occur in atmospheric conditions in such time intervals, and that would give measurements of emission in absolute terms in order to make possible quantitative estimates of constituents. The changes required in the instrumentation¹ to give these improvements were the introduction of a fast data acquisition system, the adoption of the polarised interferometric method both to suppress the effects of signal fluctuations and to allow continuous reference to a cooled black-body calibration source^{2,3}, and the incorporation of greatly improved germanium bolometric detectors (P. A. R. Ade and S. El-Atawy, personal communication).

At the altitude of the aircraft (up to about 39,000 feet) there was appreciable atmospheric water vapour. Nevertheless, most

of the spectra were recorded at an elevation of no more than 14° to give a long path for the minor constituents. The aircraft was, during most flights, close to the tropopause (high at that time of the year) and it would have been unproductive to take measurements over a range of elevation angles so as to give limb-scanning information on the vertical structure of the atmosphere (though a suitable input mirror system had been incorporated for this purpose). It was, however, essential to have a servo-controlled input mirror, taking its drive from the aircraft's inertial guidance system to maintain constant elevation during the recording of an interferogram.

The emission was continuously compared with that from a black-body cavity; to give absolute calibration two black-body cavities were used, one at ice temperature and the other at liquid-nitrogen temperature. More than 100 interferograms were recorded, and even though the analysis is not yet complete it is clear that the new techniques were successful. To illustrate this, Fig. 1 shows small parts of two of the emission spectra, relative to the ice black body. The interferogram for each was recorded in 8 min and the spectrum obtained covers the range $3\text{--}40\text{ cm}^{-1}$, of which only the part from 20.5 to 22.5 cm^{-1} is shown. The Fourier transform was linearly apodised to give a resolution of 0.02 cm^{-1} . One of the spectra is displaced upwards to avoid overlapping. Both were obtained at an altitude of 33,000 feet and at an elevation angle of 14° ; they were recorded successively. It can be seen that most features are reproduced in the two spectra. The positions of lines of possible constituents of the atmosphere are marked at the top of Fig. 1, as derived from theoretical calculations and laboratory measurements (J. W. Fleming, private communication).

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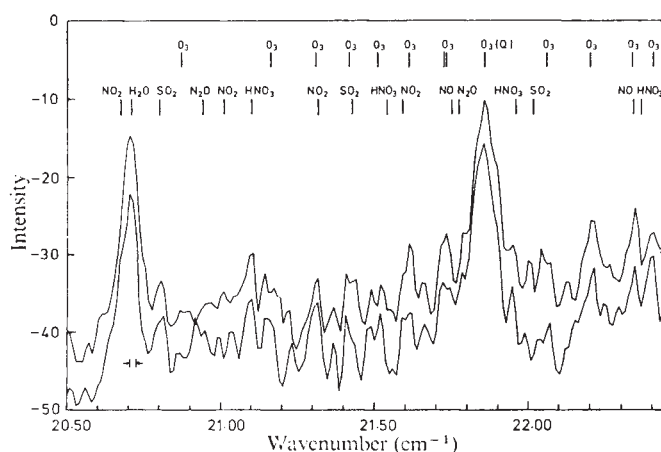
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Fig. 1 Parts of two of the emission spectra.



Photography and photometry of the near infrared OH airglow

WE are able¹ to photograph the near infrared OH airglow, and the ASSESS mission allowed us to carry out: (1) horizontal parallax measurements of airglow features over a wide geographic range, using the plane's motion to generate a baseline; (2) photometry over a wide range in latitude and longitude to



study any geographical variation in intensity; (3) correlation studies of the brightness of OH bands between 690 and 840 nm for comparison with earlier work².

Our instruments were modified for aircraft use primarily by automating the repetitive measurements and incorporating image intensifier tubes to reduce exposure times, thereby minimising smear of photographs due to aircraft roll.

Three separate instruments were flown. (1) A 35-mm camera obtained exposures every 1 min of a $25^\circ \times 35^\circ$ area of the sky. A two-stage image tube with extended S-20 photocathode, 50-mm focal length input lens, and 720-nm cut-on filter, provided usable photographic densities in 1 s. (2) Colinear with the 35-mm camera an absolutely calibrated photometer viewed the central portion of the picture. An extended S-20 phototube preceded by a 7.5 cm $f/5$ telescope and an eight-position filter wheel with 20-s dwell time comprised the photometer. Four 10-nm bandpass filters were centred on OH bands at 690, 730, 790, and 840 nm, two filters were centred on non-airglow regions at 710 and 820 nm, one filter was identical to that on the 35-mm camera, and one position was opaque so that the dark current could be measured. (3) A 16-mm movie camera photographed another image tube with input lens and filter every 2 s, giving a continuous record of aircraft roll, as well as providing a motion picture of the passing airglow structures, tropospheric clouds, stars, and the Milky Way.

During the ASSESS mission 18 h of data (representing about 97% of the time suitable for airglow studies) were obtained. The entire mission of 14 flights has provided some 3.5 h of data per flight.

We have 2,500 35-mm frames (see Fig. 1), plus 45 h of photometer data, to analyse. With these data we will study the variation of parallax emission heights with geographical location and the relationship of OH patch or stripe size to emission height. The photometer will show any correlation between OH band intensity and geographical position, as well as the correlations between band intensities. By comparing photographic and photometric data, a correlation will be sought between type of structure and/or intensity with the height of the tropopause, magnetic and/or solar activity indices, local weather, and geographical features such as mountain ranges.

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¹ Peterson, A. W., and Kieffaber, L. M., *Nature*, **242**, 321–322 (1973).

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Filter wedge spectroscopy in the region 3–6 μm

SECTRA of Venus obtained previously in the region 1–6 μm led to the identification of H_2SO_4 as the primary aerosol in its atmosphere^{1,2}. Identification depended on comparison of the detailed shapes of the calculated and observed absorption features. The ASSESS mission provided the opportunity to observe Venus at a different phase angle, providing additional data on the structure of its upper atmosphere. The chance also arose to observe IRC+10216, which has not been studied in this spectral region with an airborne telescope.

The instrument flown consisted of a continuously variable filter wedge spectrometer plus a liquid nitrogen-cooled InSb detector system. The bandpass of the spectrometer was from 2.8 to 5.6 μm with roughly 1.5% resolution and it was rotated continuously while taking spectra. Signal processing used a standard phase-lock amplifier system referenced to the oscillating secondary mirror of the telescope. The spectrometer and detector were mounted at the focus of the Meudon 30-cm telescope. The overall system bandpass, including the effects of the Mylar window, was approximately 3.5 to 5.0 μm .

Unfortunately the aerodynamic loading on the Meudon telescope was so severe as to require that a Mylar window be installed over the cavity. This compromised our data in three ways: (1) Mylar has a rather broad absorption feature centred at roughly 3.4 μm , just in the best region for studying H_2SO_4 on Venus; (2) a large offset signal due to the window was observed; and (3) condensation on the window gave some signal attenuation, though this was a minor problem in this spectral region. Nevertheless, spectra were obtained of Venus, IRC+10216, and the Moon for calibration and are being analysed.

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Airborne television observations of airglow clouds in the near infrared

AN image iscocon intensifying television system was flown to make a survey of the size, geographical distribution and frequency of occurrence of airglow clouds in the near infrared. Peterson and Kieffaber¹ have published photographs of cloud-like structures identified as OH airglow, taken at low elevation angles and with exposures of several minutes, on clear nights in New Mexico. The aim of our experiment was to establish whether airglow structures detected with the image intensifying television could be used as tracers of winds in the upper atmosphere.

The television system can detect very faint light signals (it provides at least two orders of magnitude intensification), with the option of integrating the image inside the television tube for periods from 1/25 s up to several seconds. This instrument could therefore detect and record airglow emission structures rapidly and so give good spatial and temporal resolution. Furthermore, the use of an aircraft as an observation platform makes it possible to eliminate most of the interference from meteorological clouds.

The television camera was primarily positioned looking out at 60° to the horizon, but on two flights it was situated at a 15° window. The camera had a 30° field of view. The long wavelength cut-off at the television tube was ~ 900 nm, with peak response at ~ 600 nm. A Wratten filter with short wavelength cut-off at 670 nm was placed in front of the camera lens. Peterson and Kieffaber² made observations of airglow clouds



Fig. 1 Airglow clouds looking east from an altitude of 39,000 feet on June 18 at 0930 UT (0122 LT), latitude 45°, longitude 122°W. The picture was obtained by integrating over seven television fields, that is, for 0.28 s.

at a 15° window on the same side of the aircraft throughout the mission.

We found that well defined cloud-like structures could be observed easily with the camera only from the low elevation (15°) window (see Fig. 1). The atmosphere was not sunlit below about 400 km at this position, thus excluding the possibility that these structures are noctilucent clouds.

Assuming that the aircraft speed (~ 250 m s⁻¹) is likely to be much greater than upper atmospheric wind speeds at middle latitudes, we can estimate the altitude (80–85 km) and horizontal dimension (~ 20 km) of individual bright patches of airglow.

Airglow viewed through the 60° window was diffuse, and small scale structure was not easy to identify. There were, however, variations in intensity over the television field of view, particularly when well defined structures were visible at lower elevations through the image intensifiers of the cameras of the New Mexico group. Identification of individual clouds was complicated by the aircraft roll during the longer integration times (1 or 2 s) needed at the high elevation window because of lower airglow intensities. These lower intensities indicate that the thicknesses of the airglow structures are small compared with horizontal dimensions. The general airglow brightness, seen from the 60° window, varied from day to day and from place to place; we are making a separate study of these variations from the television records.

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- ¹ Peterson, A. W., and Kieffaber, L. M., *Nature*, **242**, 321 (1973).
² Peterson, A. W., and Kieffaber, L. M., *Nature*, **257**, 649–650 (1975).

Far infrared observations of dark cold clouds and H II regions

WE present here a brief description and preliminary results of our airborne far infrared experiment (see ref. 1). At the focus of a 30-cm open-port Cassegrain telescope was a Low-type germanium bolometer, NEP of 3×10^{-14} W Hz^{-1/2}, following a four-filter set of bandwidths, respectively, 31–38 μ m, 47–67 μ m, 72–94 μ m and 114–196 μ m. Cold diaphragms from 1.57' to 6.3' were actually used². The secondary mirror was wobbling at 37 Hz, with a typical beam throw of 9', and scanning in both x and y was possible up to 30' $\times$ 30'. The stability of the system was better than 1' and was as low as 15'' during steady parts of the flights. Tracking was done by means of the star video signal (from a Nocticon tube), which was electronically processed and looped on the torque motors of the gyro-stabilisation. A PDP-11 computer was used for on-line visualisation of digitised maps.

Calibration was carried out seven times on Venus, but unfortunately not for all the flights reported on here. Significant and variable 'sky noise' (of up to 20 times the detector noise) meant that only some of the data is really usable; we therefore made systematic measurements on this 'sky noise' (to be published).

Table 1 Flux intensity in 10^{-23} W m ⁻² Hz ⁻¹			
Source	114–196 μ m	72–94 μ m	46–67 μ m
W51	4.4 ± 0.5	16 ± 2	42 ± 4
M17	28 ± 8	—	—
ρ Oph	14 ± 4	—	—
S131	22 ± 6	—	—

The aim of the experiment was the detection and mapping at several wavelengths of dark galactic clouds. Four sources were studied: ρ Ophiuchus, S131, W51 and M17. At this early stage of the data examination, only the last two seem to show extension and structure. Table 1 summarises the fluxes computed for these four objects assuming, for Venus, a black-body temperature³ of 220 K, and a size of 19'. We can derive for W51 an equivalent black-body temperature significantly higher than that proposed by Harvey⁴, at least 100 K. As for M17, our values agree well with those of Hoffmann⁵, assuming that the source is extended and uniformly bright. Our studies of ρ Oph and S131 confirm the high degree of correlation between CO clouds and dark regions^{6,7}.

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Operon structure of DNA transfer cistrons on the F sex factor

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The operon structure of the tra cistrons on the F sex factor has been investigated using Mu-1 bacteriophage. Twelve tra cistrons comprise one polycistronic operon 15 × 10⁶ to 20 × 10⁶ daltons long.

GENETIC analyses¹⁻⁷ of transfer-deficient (Tra⁻) mutants of the F sex factor have revealed the existence and map order of thirteen *tra* cistrons on F as summarised previously^{8,9}. Electron microscopic analysis^{10,11} of DNA heteroduplexes made with Tra⁻ mutants, has resulted in a physical map that correlates with genetic assignments as shown in Fig. 1. The physical units are kilobases (kb), an abbreviation for one thousand nucleotide bases or base pairs. Throughout this article, right and left refer to the (arbitrary) orientation used in Fig. 1. The *tra* cistrons are clustered according to function in that cistrons between 62 and 79 kb are involved in F pilus synthesis while cistrons between 80 and 94 kb have other DNA transfer-related functions but are not necessary for F pilus synthesis. We believe that *traJ*, located to the left of all other *tra* cistrons, codes for a positive control protein, necessary for the production of all other *tra* cistron gene products; thus *traJ*⁻ mutants lack all the other *tra* gene products as demonstrated by genetic tests¹².

One of the *tra* cistrons, *traS*, lies between 80 and 89 kb (between *traG* and *traD*⁷). *traS* codes for the surface exclusion phenomenon wherein F-carrying cells are poor recipients (Sfx⁺) for conjugation with other F-carrying donor cells. *traS*⁻ mutants are still transfer-proficient²⁰. Thus the wild-type F factor exhibits a Tra⁺ and Sfx⁺ phenotype. *traJ*⁻ mutants are Tra⁻ and Sfx⁻, whereas mutants in other *tra* cistrons are Tra⁻ but Sfx⁺. A few nonsense and frameshift mutants located to the left of *traS*,

are partially surface-exclusion-deficient and exert polarity on *traS* as if it were in the same operon². None of these putative polar mutants, however, lies to the left of *traK* (68–73 kb), so they are of little use for defining the operon structure of all the *tra* cistrons. The genetic properties of Tra⁻ deletion mutants³ indicated, however, that any polycistronic *tra* operon(s) must be transcribed from left to right. Accordingly, we initiated a search for absolute polar mutants mapping in each of the *tra* cistrons, to investigate their polarity on the other *tra* cistrons and thus to define the structure of the *tra* operon(s). Mu-1 bacteriophage has been found to insert itself randomly within *Escherichia coli* DNA¹³ and such Mu-1 insertions manifest (absolute) polarity on any cistrons lying distal to the Mu-1 insertion site¹⁴⁻¹⁸ in the direction of transcription by acting as a transcriptional block¹⁹. Indeed if all the *tra* cistrons between *traA* and *traI* were in one operon, the *traA*–*traI* operon would have a length of 15 to 20 × 10⁶ daltons and thus would be the largest bacterial operon yet described.

Isolation of Mu-1 insertion mutants

The information we have on *traS* predicted that we could separate Mu insertions into two classes on the basis of whether or not they were polar on *traS*. Mu insertions polar on *traS* would lead to an Sfx⁻ phenotype, whereas Mu insertions not polar on *traS*, because they were in a different operon or operator-distal to *traS*, would be Sfx⁺. The isolation of Sfx⁻ mutants, however, is technically difficult for the following reasons. Good recipient mutants (Sfx⁻) are at a growth disadvantage in a mixed population with Sfx⁺ wild type cells because of the repeated unidirectional DNA transfer from wild type cells to Sfx⁻ mutants which results in a relative dilution (contraselection) of the

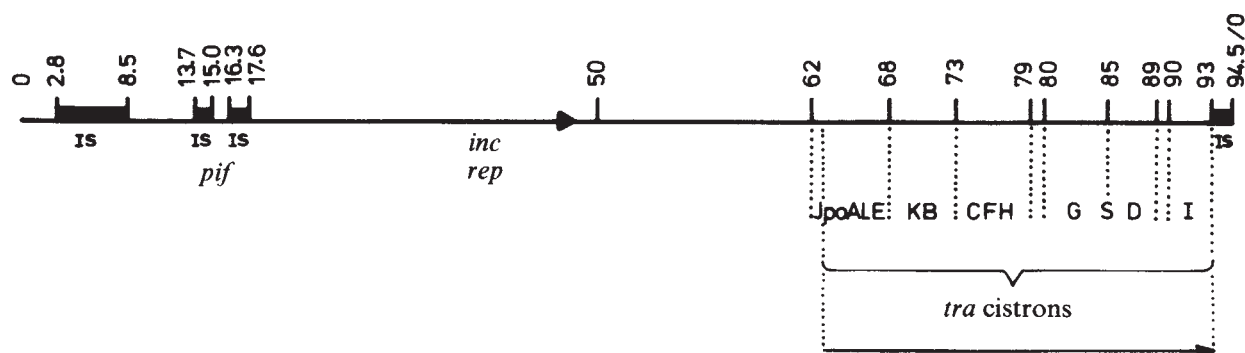


Fig. 1 The physical map of F. All units given are in kilobases (kb) according to the results of Davidson *et al.*¹¹. IS represents the known locations of insertion sequences¹¹. The location of the *pif* cistrons which code for restriction of T7 and ϕ_{11} phages is according to M. Malamy (personal communication). The location of the *rep* cistron(s) which code for vegetative replication is according to Davidson *et al.*¹¹ while the location of the *inc* cistron(s) which code for incompatibility is according to Willetts⁷. The location of the origin of DNA transfer is represented as an arrowhead and is according to Davidson *et al.*¹¹. The location of the *tra* cistrons are according to the map order defined genetically³, the correlation of genetic results between different sets of mutants by Willetts and Achtman², and the correlation of genetic results with physical results by Davidson *et al.*¹¹. The *tra* cistrons listed are known to map between the dotted lines on the basis of the length of genetically characterised deletions. *traS* lies between *traG* and *traD*⁷ but its exact location is not yet known. The location of *traL* is according to the results presented by Willetts⁶ correlated with the results presented here. The locations of the promoter (p) and operator (o) are according to the results presented here. The arrow under the *tra* cistrons depicts the transcriptional direction of the *tra* operon as demonstrated here.

Table 1 Genetic locations of Mu-1 insertion mutants

Method	J	A	E	K	No. of mutants in		F	H	G	S	D	I	Total
					B	C							
1	0	0	0	0	0	0	3	5	0	0	10	1	19
2	0	3	2	0	7	2	9	7	2	0	0	0	32
3	1	6	0	0	0	0	0	0	0	0	0	0	7

For all three methods, Mu-1 lysates were spotted on bacterial cultures of (JCFLO)⁺ derivatives of strains JC3272 (methods 2 and 3) or JC5455 (method 1)²⁴. JCFLO is a *lacI*⁻ derivative of *Flac* (F42)²⁴. After 4–6 h at 37 °C for lysogenisation, subcultures were inoculated in nutrient broth with 0.01% SDS (methods 2 and 3) or without SDS (method 1). Overnight growth at 37 °C was conducted to allow segregation and expression. For method 1 dilutions were plated and the resulting colonies screened for Tra⁻ mutants by replica-plate crosses²⁴ against a *thr::Mu-1* F⁻ recipient culture. For methods 2 and 3, the overnight cultures were diluted into nutrient broth without SDS, grown to exponential phase and mated with a Mu-1 resistant derivative of Hfr KL98. His⁺ (Str^R) recombinants were selected on minimal medium plates containing SDS. For method 2, the His⁺ recombinants were screened for Tra⁻ and Sfx⁻ mutants by replica-plate crosses²⁷ against *thr::Mu-1* derivatives of an F⁻ recipient and an (F8)⁺ donor strain, respectively. For method 3, replica-plate crosses were first used to transfer R100-1 from a Mu-1 resistant (R100-1)⁺ donor culture to the His⁺ recombinants and these (R100-1) derivatives were then screened for M12-resistant clones. All the mutants listed were isolated from independent cultures and are of independent origin. A further 33 mutants isolated by method 2 are described in the text.

Sfx⁻ mutants. By incorporating 0.01% sodium dodecyl sulphate (SDS) in liquid and solid media, mating pair formation can be prevented, thus facilitating the isolation of Sfx⁻ mutants²⁰. The procedure used (ref. 20) consisted of including SDS in all growth media to prevent loss of Sfx⁻ mutants and included an enrichment for Sfx⁻ mutants by selecting recombinants after a mating between the mutagenised population and an Hfr donor. The recombinants were enriched approximately 100-fold for Sfx⁻ mutants compared with the (poor recipient) Sfx⁺ wild type.

The above information enabled us to design three mutant isolation procedures intended to yield (Mu-1)⁺ mutants which were, respectively, Tra⁻ Sfx⁺, Tra⁻ Sfx⁻ and *traJ*⁻. In the first procedure, we screened 39,000 (Mu-1)⁺ lysogens of an (F_{lac})⁺ strain for Tra⁻ mutants after an overnight growth step in the absence of SDS. As expected, a number of Sfx⁺ mutants (11/19) was isolated, in addition to eight Sfx⁻ mutants which escaped the natural selection against such mutants during growth of mixed populations. For the second procedure, SDS was present in all growth media to prevent loss of Sfx⁻ mutants and these mutants were then enriched by a mating with an Hfr donor culture followed by selection of recombinants. SDS was omitted during the mating. We screened 13,000 recombinants of (Mu-1)⁺ lysogens of an (F_{lac})⁺ strain and 65 Tra⁻ Sfx⁻ mutants were isolated. The first 32 analysed genetically by complementation tests seemed to represent an adequate sample of the *tra* cistrons except *traJ*, and the remaining mutants were tested only to ensure that none carried a *traJ*⁻ mutation. The complementation tests of the 65 mutants just described demonstrated that each mutant gave poor complementation for one or more clustered cistrons. We assigned a tentative genetic location for each Mu-1 insertion as being in or immediately to the left of the left-most cistron which was poorly complemented (Fig. 1). This assignment is demanded by the results of complementation tests with deletion mutants³ which demonstrated that any

polycistronic operons are transcribed from left to right. The genetic locations of the mutants are presented in Table 1. No *traJ*⁻ mutant was isolated by procedures 1 or 2 and the complementation results (discussed in detail below) did not enable any conclusion to be drawn about whether or not *traJ* was in the same operon.

The third procedure took advantage of the special properties of *traJ* and *traA*. As described above, *traJ*⁻ mutants lack the gene products of the other *tra* cistrons including *traA*^{12,21}. Willetts²¹ has shown that cells carrying either a *traJ*⁻ mutant or a *traA*⁻ mutant of F, plus the sex factor R100-1 (which has considerable DNA homology with F within the *tra* region²²) are relatively resistant to M12 male-specific bacteriophage, while cells carrying R100-1 and an F factor mutant in any other *tra* cistron are sensitive. Although the mechanism of this phenomenon is still not totally clear, we took advantage of it as a screening procedure for *traJ*⁻ mutants. We thus repeated the procedures used in the second method but in addition a strain carrying the R100-1 factor was mated with the selected recombinants; (R100-1)⁺ progeny were screened for M12-resistant clones by replica plating methods. The corresponding non-R100-1 containing colonies on the master plates were purified and analysed. Seven mutants were isolated out of 200,000 recombinants of which six carried Mu-1 in *traA* and one carried Mu-1 in *traJ*.

All the mutants listed in Table 1 have been shown to carry Mu-1 on the F factor since after acridine orange curing²³ to remove the F factor, the ability to produce Mu-1 phage was concurrently lost. All 58 mutants in Table 1 were tested for their DNA transfer ability and their surface-exclusion ability by replica-plating methods and in 33 out of 58 cases, chosen as representative of the various *tra* cistrons, by quantitative measurements as well. The qualitative and quantitative results (Table 2) were essentially the same as those described for deletion mutants^{3,7} which contained sequentially longer deletions

Table 2 Properties of Mu-1 insertion mutants

Mu location	No. of mutants	TE*	SEI†	F ₁	F ₂	Qβ ³
<i>traI</i>	1	3 × 10 ⁻⁷	67	S	S	S
<i>traD</i>	3	1 × 10 ⁻⁵	≥ 200	S	R	S
<i>traD</i>	1	1 × 10 ⁻⁵	46	S	R	S
All other cistrons	27	1 × 10 ⁻⁶	1–3	R	R	R
<i>traH</i>	1	1 × 10 ⁻⁶	13	R	R	R

S, Sensitive; R, resistant.

*The transfer efficiency²⁴ (TE) is defined as the number of (F_{lac})⁺ exconjugants derived from the F⁻ parent which are formed in a mating between the mutant strain used as donor and a suitable F⁻ recipient. The value is normalised for the number of mutant cells added to the mating mixture.

†The surface exclusion index²⁴ (SEI) is obtained by dividing the number of recombinant colonies measured in a mating between an Hfr and an F⁻ culture by the number measured in a parallel mating between the Hfr and a test culture. A value of 0.5 to 1.0 for the TE and 100 to 200 for SEI are typical of a wild type (F_{lac})⁺ culture.

Table 3 Conjugational complementation tests with reference mutants

Location of mutation	Sex factor	Donor mutation								
		<i>traJ90</i>	<i>traA1</i>	<i>traE18</i>	<i>traB2</i>	<i>traC5</i>	<i>traF13</i>	<i>traH80</i>	<i>traG24</i>	<i>traD14</i>
J	MPFL10	< 0.008	0.8	0.6	1.3	0.6	1.6	0.4	0.5	1.2
A	MPFL6	0.75	0.01	0.01	0.008	0.03	0.2	0.03	0.01	0.2
E	MPFL5	1.9	0.6	0.07	0.04	0.02	0.4	0.3	0.3	0.4
B	MPFL4	1.2	0.4	0.9	0.03	0.04	0.3	0.1	0.09	0.4
C	MPFL11	1.2	0.6	0.9	1.0	0.03	0.04	0.1	0.03	0.07
F	JCFL171	1.0	1.2	1.3	2.3	1.4	0.01	0.02	0.05	0.01
H	JCFL161	2.8	1.9	1.9	3.1	2.6	1.3	0.02	0.1	0.05
G	JCFL184	0.7	1.9	1.6	3.0	1.6	2.6	1.9	0.005	0.09
D	MPFL2	0.8	1.2	2.0	1.0	1.1	0.3	0.7	0.9	0.03
Right of D	JCFL146	1.1	2.5	3.2	2.5	2.8	0.8	1.6	3.7	1.4

These results were obtained with complementation tests performed 144 at a time in Microtiter plates as before^{1,23} but with an improved normalisation method. The values representing poor complementation are underlined. Each test consists of two successive crosses in which first an amber-suppressible *tra* mutant was introduced from a Su^+ host into the Su^- (Mu-1)⁺ insertion mutant strains. The degree of complementation was measured in the second cross by measuring the transfer efficiency of this population to an F^- (Mu-1)⁺ recipient after first killing the Su^+ primary donor with T6 phage. Normalisation was possible by always including an *Flac tra^+ lacZ^-* mutant (amber *lac* mutation) as donor and a different *Flac tra^+ lacZ^-* mutant (temperature-sensitive *lac* mutation) as recipient in every set of crosses. When F transfer by complementation due to the *tra^+ lacZ^-* donor was tested, a $Su^- F^-$ host was used in the second cross so that transfer of only the complemented mutant was measured. When F transfer by complementation due to the *tra^+ lacZ^-* recipient was tested, a $Su^+ F^-$ host was used in the second cross (transfer of the *Flac lac^-* mutant itself is not seen since these cells are *Lac^-* at the incubation temperature of 37 °C). The use of these two *Flac lacZ^-* mutants enabled us first to normalise the various donor cultures against the *lacZ^-* recipient mutant where all are complemented, and second to normalise the various recipient cultures against the *lacZ^-* donor mutant which also complements all of them. These two normalisations remove any effects due to different recipient ability of the individual recipient cultures and to different donor ability of the individual donor cultures.

running from *traL* leftwards. All mutants mapping to the right of *traS* were Sfx^+ and Tra^- and made F pili. All mutants mapping to the left of *traS* were Sfx^- and Tra^- and male specific phage resistant. The mutants tested only by replica-plate methods yielded qualitatively identical results.

The possibility existed that Mu-1 was not integrated at the site of the *tra* mutation, but elsewhere on *Flac* in the various mutants. This possibility was tested for a representative group of the mutants by using transduction with P1 phage to introduce *tra^+* DNA from a wild-type *Flac* element, followed by a mating to select Tra^+ recombinants. The mating was performed at 33 °C with a lysogenic F^- recipient with a temperature-sensitive Mu-1_{ets62} prophage integrated in *thr*. Since the recipient is immune to Mu-1 phage at 33 °C, both *Flac* elements either carrying Mu-1 or not carrying Mu-1 will establish themselves after transfer, and zygotic induction will not occur. At 42 °C the immunity due to the chromosomal Mu-1 prophage disappears and the cells die due to temperature induction unless the *Flac* element carries a Mu-1 prophage. Thus we could determine how often Tra^+ recombinants that were still lysogenic for Mu-1 were formed. Fifty colonies were tested from each cross: in all cases the Tra^+ colonies did not contain Mu-1 on *Flac* and the corresponding mutants must therefore carry Mu-1 inserted at the site of the *tra* mutation.

Operon structure

Table 3 presents results of complementation tests, using the conjugational complementation method¹ with the same

representative mutants mentioned above. Each Mu-1 insertion is polar on the neighbouring cistrons to the right regardless of where Mu-1 is inserted, except for Mu-1 in *traJ*. Furthermore, most of the Mu-1 insertions showed clear polarity on all the cistrons to the right of the insertion, although in many cases the polarity was not absolute. The Mu-1 insertion in *traJ* showed no polarity effects. The polarity pattern indicates first, that the direction of transcription for *traA* to *traD* is from left to right (Fig. 1) in agreement with results from deletion mutants³. Second, the 11 cistrons *traA* to *traD* must lie in one operon. These results also agree with the properties of three polar *traK* mutants, one polar *traC*, and one polar *traH* mutant which showed polarity through to *traS*^{1,2,20}. In addition 15 other amber, ochre, UGA or frameshift mutations, lying between *traB* and *traS* have also been shown to exert some polarity on *traS* (unpublished observations of M. A. and N. Willetts).

The results in Table 3 also indicate some residual complementation in tests between Mu-1 insertion mutants and mutations located distally in the *tra* operon. Such complementation was not observed in tests between deletion mutants and the same point mutations³ and therefore this low level of complementation demonstrates that the Mu-1 insertions have not deleted the cistrons being tested. This residual complementation is discussed further below.

The cistrons *traL*, *traK* and *traI* are not testable by the method described above for technical reasons, and so these three cistrons were tested by the P1 transductional method² (Table 4). These results taken together with those of

Table 4 P1 transductional complementation analysis with reference mutants

Location of Mu-1	P1kc lysate from	% complementation with recipient mutation		
		<i>traL311</i>	<i>traK105</i>	<i>traI65</i>
<i>traJ</i>	MPFL10	35	10	16
<i>traA</i>	MPFL6	0.05	< 0.01	< 0.1
<i>traE</i>	MPFL5	86	< 0.01	< 0.1
<i>traB</i>	MPFL4	101	9	< 0.1
<i>traC</i>	MPFL11	112	96	< 0.1
<i>traF</i>	JCFL171	78	73	< 0.1
<i>traH</i>	JCFL161	167	6	< 0.1
<i>traG</i>	JCFL184	118	11	< 0.1
<i>traD</i>	MPFL2	199	257	< 0.1
<i>traI</i>	JCFL146	179	272	0.3

These tests were performed as described previously² except that P1kc was used instead of P1*virA*. Negative values are underlined. P1kc lysates grown on the (Mu-1)⁺ insertion mutants were used to transduce strains carrying an *Flac* mutant carrying either *traL311*, *traK105* or *traI65* mutations. Complementation of the resulting abortive transductants was then assayed by crosses with an F^- strain. All results were normalised to a value of 100% for a P1kc lysate grown on a wild type *Flac* element after previous normalisation for the few colonies obtained due to leakiness in control transductions with a P1kc lysate grown on an F^- strain.

Willetts define the map location of *traL* as being between *traA* and *traE*. Furthermore Mu-1 insertions between *traA* and *traD* exert polarity on *traL*, *traK* and *traI* only when they lie to the left of that cistron. The Mu-1 insertion in *traI* did not manifest any polar effects.

The conclusion that the *tra* operon extends to *traI* was reinforced by the results of a third method. Willetts²¹ has shown that *traI*⁻ and *traI*⁻ point mutants are not complemented for DNA transfer by the related sex factor R100-1 although point mutants in all other *tra* cistrons are complemented. Tests of (R100-1)⁺ derivatives of point mutants and of Mu-1 insertions in *traJ*, *traA*, *traC*, *traD* and *traI* confirmed these conclusions for the point mutants but indicated that all the Mu-1 insertions were not complemented by R100-1. These results are those expected if Mu-1 insertions between *traA* and *traD* exert polarity on *traI*. Thus all the cistrons between *traA* and *traI*, inclusive, form one operon transcribed in the direction *traA* to *traI* and henceforth referred to as the *tra* operon. *TraJ* must be part of a second operon, whose transcriptional direction is unknown.

The residual complementation shown in Table 3 in crosses where polarity was present might be due to reinitiation within the *tra* operon, to Mu-1 insertions not being absolutely polar, or to the formation of Tra⁺ recombinants. The complementation seen is, however, at the limit of resolution of the method used for Table 3. The nature of the complementation was therefore investigated by a much

traH, *traG*, *traS*, *traD*, and *traI* lie in one polycistronic operon. These conclusions are strengthened by the properties of other polar mutants available which were isolated after nitrosoguanidine or ethyl methanesulphonate treatment^{1,2,20,24}. Figure 1 shows that this operon has a maximal length of 93-62=31 kb. This stretch, however, includes *traJ* which is in a second operon. Since *traI* is to the right of 90 kb and *traE* is to the left of 68 kb, a minimal estimate is provided by assuming 0.3 kb for *traI* and 1 kb for *p o traA traL traE* yielding 90-68+1.3=23 kb. Thus the operon is 23-31 kb (or 15×10⁶-20×10⁶ daltons) long. If the mRNA is synthesised as one continuous piece it would be approximately 7×10⁶-10×10⁶ daltons long and take 7-10 min to transcribe at the rate of 55 nucleotide base pairs per second²⁵.

traA through to *traH* code for the synthesis of F pili and *traA* has been found to code for the F pilus subunit (personal communication from C. C. Brinton, Jr). *traG*, *traD* and *traI* mutants synthesise F pili and even form mating pairs with F⁻ cells²⁴. No DNA transfer ensues, however, from these mating pairs and *traG*, *traD* and *traI* probably code for enzymes involved directly in DNA transfer. *traS* codes for surface exclusion and expression of *traS* prevents DNA transfer from Tra⁺ cells²⁶. Thus, although these cistrons code for different functions, they are all DNA transfer-related.

Surface exclusion has a strong evolutionary advantage since the Tra⁺ Sfx⁻ mutants that we have isolated are

Table 5 Conjugational complementation tests in Rec⁺ and Rec⁻ hosts

Recipient Flac sex factor	Location of Mu-1	Fhis donor							
		Host	Tra ⁺	<i>traJ90</i>	<i>traA1</i>	<i>traE18</i>	<i>traB18</i>	<i>traF13</i>	<i>traD83</i>
MPFL6	A	Rec ⁺	0.94	0.37	≤ 0.0004	≤ 0.001	≤ 0.0003	0.005	0.03
MPFL6	A	Rec ⁻	0.79	0.39	≤ 0.0003	0.0009	0.0009	0.0007	0.005
MPFL5	E	Rec ⁺	0.20	0.16	0.05	0.005	0.05	0.004	0.07
MPFL5	E	Rec ⁻	0.90	0.68	0.14	0.007	0.0003	0.002	0.01
JCFL184	G	Rec ⁺	—	1.4	0.68	0.20	0.60	0.62	0.005
JCFL184	G	Rec ⁻	—	0.96	1.51	1.61	3.22	0.36	0.004

Tube complementation tests¹ were performed using transient heterozygote cells carrying both *Fhis tra*⁻ and *Flac tra::Mu-1* mutants. The transfer efficiency per heterozygote RecA⁺ or RecA⁻ cell is shown. The Rec⁺ hosts were the same strains used in Table 2 whereas the Rec⁻ hosts were closely related *recA58* derivatives.

more sensitive tube complementation method¹. For these tests the *Flac* Mu-1 insertion mutants were transferred to a RecA⁻ host. Furthermore, *Fhis tra*⁻ mutants were isolated after recombination between *Fhis* (F57¹) and the appropriate *Flac tra* mutants containing point mutations. The genetic character of both the new *Fhis tra* mutants and the transferred *Flac* Mu-1 insertion mutants were confirmed with the same methods as used for Table 3.

Fhis/Flac heterozygote cells can be assayed by the formation of Lac⁻/Lac⁺ sectorised colonies and thus it is possible to estimate directly the complementation efficiencies per heterozygote cell. The results of such tests are presented in Table 5. Other tests between *Flac* mutants with Mu-1 in *traF*, *traH*, *traB* and *traD* and mutants carrying *Fhis traA1* or *traD83* were also performed with comparable results (data not shown). The conclusion from all these tests is that some residual complementation is in fact seen in both RecA⁺ and RecA⁻ hosts in crosses where polarity should result in no complementation. Complementation was accompanied by recombination in the Rec⁺ host but no Tra⁺ recombinants were formed in any of the crosses in the RecA⁻ host (data not shown). Thus either Mu-1 insertions are not absolutely polar in this system or there is some low level reinitiation within the *tra* operon.

Implications

The data presented here on Mu-1 insertion mutants demonstrate that *traA*, *traL*, *traE*, *traK*, *traB*, *traC*, *traF*,

unstable in normal growth conditions²⁰. This instability would result in the selection of Tra⁺ Sfx⁺ mutants from a primaeval Tra⁺ Sfx⁻ population in nature in conditions where transfer was possible. It has possibly also been this selection pressure which resulted in the incorporation of *traS* into the *tra* operon since surface exclusion is thus expressed whenever the potential for DNA transfer is expressed.

The elucidation of the *tra* operon structure also simplifies any models to explain the mechanism of action of the *traJ* gene product. *traJ* is a positive control gene^{1,2} and *traJ*⁻ mutants lack the gene products of all the other *tra* cistrons¹². A simple model to explain these relationships is that the *traJ* gene product is necessary for transcription of the *tra* operon. In the case of the F factor, *traJ* is always expressed as is the *tra* operon. Most R factors are repressed²⁶ and in at least the case of R100 the mechanism seems to be by the prevention of the synthesis of the *traJ* gene product^{12,27,28}. Thus R factors can provide economy and repress the formation of sex pili, DNA transfer enzymes and surface exclusion by indirectly repressing the *tra* operon in a cascade fashion. There are always a few non-repressed cells in a repressed population which can transfer R factor DNA immediately on contact with R⁻ cells. After transfer to the R⁻ cells the *traJ* gene product is synthesised before the repressor²⁸, the *tra* operon is turned on and the new R⁺ cell can itself transfer R factor DNA to any other R⁻ cells. Thus exponentially increasing DNA

transfer occurs when a repressed R^+ population is mixed with an R^- population. As soon as all the cells have received the R factor, surface exclusion prevents further DNA transfer. In the absence of DNA transfer no new *traI* gene product is synthesised due to the repressor and dilution of the *traI* gene product eventually leads to a newly repressed population in which neither *traI* nor the *tra* operon are expressed.

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letters to nature

Discovery of powerful transient X-ray source A0620–00 with Ariel V Sky Survey Experiment

ON August 3 a faint new X-ray source was discovered (at 15 Ariel counts s^{-1}) near the boundary of the Monoceros and Orion constellations and 6° from the galactic plane, during routine monitoring of the Milky Way with the Ariel V Sky Survey Experiment (SSE). During the following week, the source—designated A0620–00—increased

rapidly in intensity to become the brightest in the sky (at 2–18 keV). The X-ray light curve from continued observation with the SSE to August 16 is shown in Fig. 1. The ordinate is in Ariel counts s^{-1} and for comparison, the corresponding count rates for the Crab Nebula and Sco X-1 are 400 s^{-1} and 3,200 s^{-1} , respectively. The precursor peak on August 6 is real and is qualitatively similar to the feature seen¹ in the rise to maximum intensity of the earlier Ariel transient A1524–62. Subsequent observations of A0620–00 with the Ariel V experiments pointing along the spin axis of the spacecraft have shown the intensity to have remained very high at least until August 27.

Figure 2 shows a copy of the Palomar Observatory Sky Survey chart marked up with the initial SSE X-ray source position, an improved Ariel V location² and the optical counterpart³ discussed in the following letter⁴. Figure 3 compares an expanded blue print of the region from the Palomar survey and a post-transient blue photograph taken with the UK Schmidt telescope at Siding Spring. In spite of the considerably reduced exposure of the new plate, limited by sky brightness—since A0620–00 is at present visible only near twilight—the considerable brightening of the optical star ($\Delta M_B \sim 8$) is readily apparent. Detection of a short lived radio source coincident with the optical star is reported in a later letter⁵.

Most current theories of optical novae^{6,7,8} invoke unstable mass transfer in a close binary system in which the accreting star is probably a white dwarf. The transient nature of A0620–00 indicates that this may have a similar origin, but the high X-ray luminosity strongly suggests a qualitative difference from optical novae (none of which have been detected by, admittedly incomplete, X-ray surveys), probably in that for an 'X-ray nova' to occur the accreting object has to be a neutron star or black hole. In the present case it is of particular importance to determine the distance and thus the X-ray luminosity of A0620–00. If, as seems likely, the source reached a maximum intensity from August 12 corresponding to an accretion rate at the Eddington limit, then the measured X-ray flux above 1 keV ($\sim 650 \text{ keV cm}^{-2} \text{ s}^{-1}$) corresponds to a limiting X-ray luminosity ($1.3 \times 10^{38} \text{ erg s}^{-1}$) for a secondary of mass M_1 at a distance of 1 kpc. A preliminary analysis of high dispersion spectra of the optical star (Dr T. Gull, personal communication), reported to show marked interstellar absorption features⁹, gives a minimum distance of 600 pc.

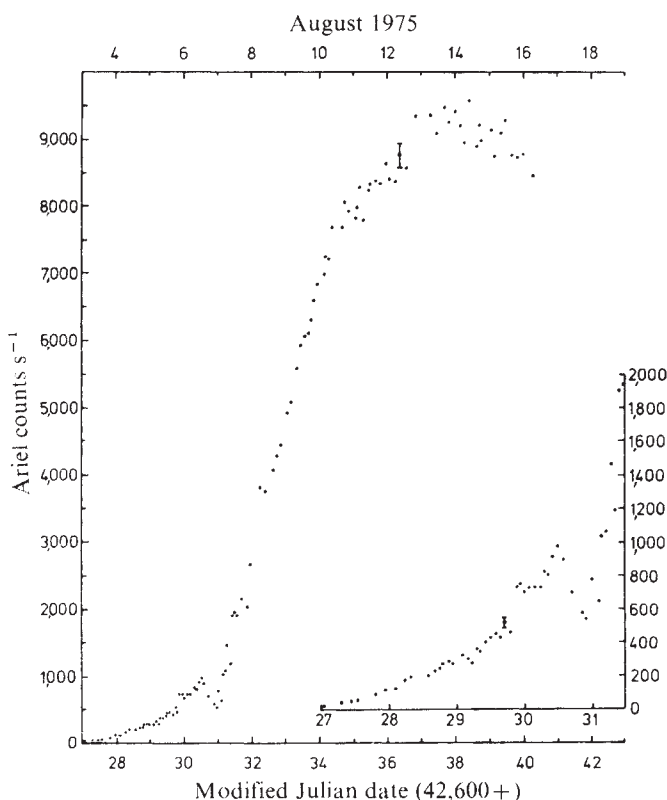


Fig. 1 The X-ray light curve of A0620–00 obtained with the SSE at 2–18 keV. The error bars are $\pm 1 \sigma$ statistical errors at the lower intensity end (see inset); towards the peak the major error arises from uncertainties of 0.1° in the spacecraft attitude.

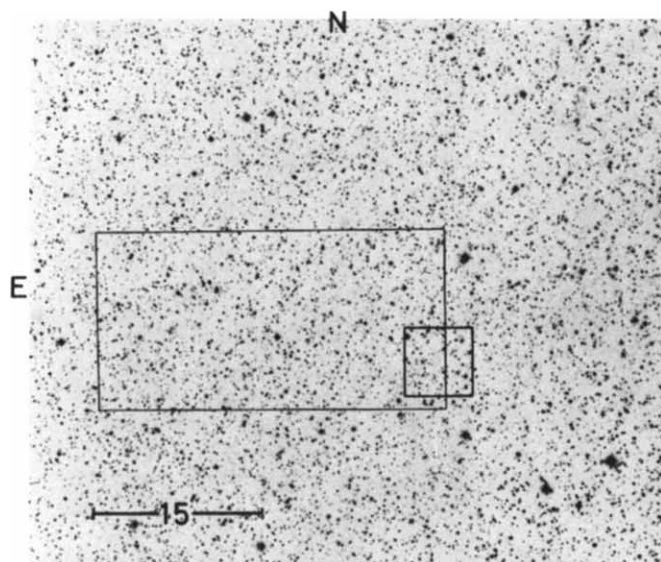


Fig. 2 Palomar Observatory Sky Survey red photograph showing the initial and improved Ariel V X-ray error boxes (90% confidence level) and the subsequently identified optical counterpart (copyright: National Geographic Society, Palomar Observatory Sky Survey).

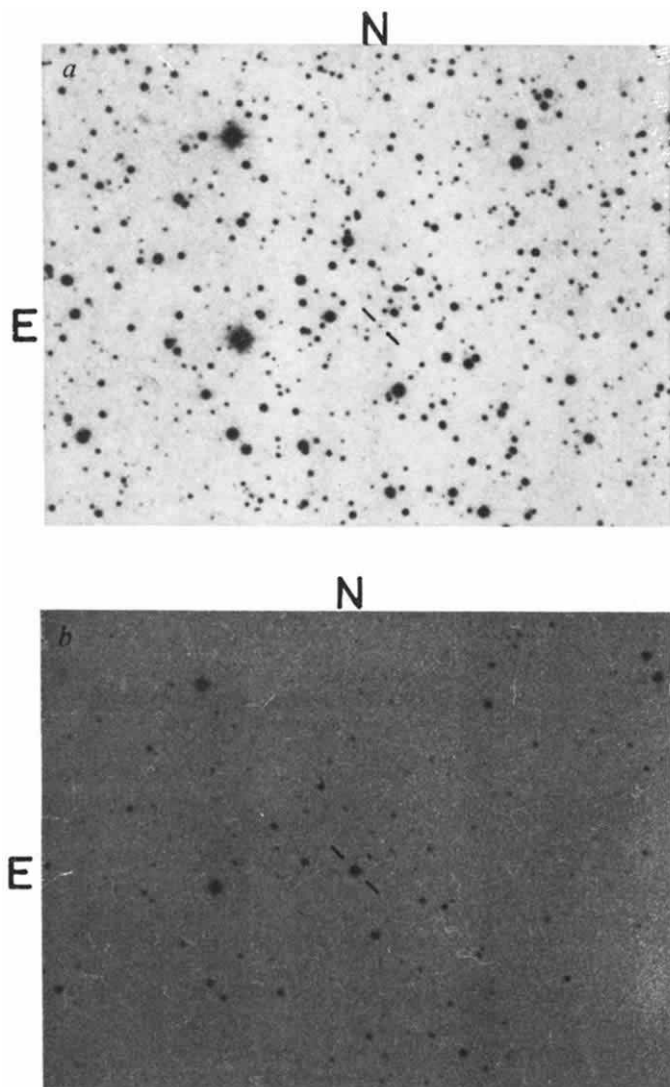


Fig. 3 A comparison of the optical counterpart on the blue POSS photograph with a post-transient blue plate taken with the UK Schmidt telescope at Siding Spring (courtesy: Science Research Council, UKSTU).

whereas the discovery of an earlier optical outburst of A0620-00 in 1917 (ref. 10) has been interpreted in terms of a recurrent nova which, if of typical luminosity for a 60-yr period nova, would indicate a distance of 5,700 pc. If confirmed, a distance of this order would imply that the accreting component of A0620-00 is a massive black hole.

Finally, a search for periodic variation in the X-ray intensity of A0620-00 has shown no modulation ($>3\%$) over time scales of 200 s - 2 d.

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The X-ray spectrum of A0620-00

A REMARKABLE change in the X-ray spectrum of the transient source A0620-00 (ref. 1) was observed by the Ariel V Sky Survey Experiment (SSE) during the rise to maximum brightness. The SSE detector being used has an effective area (behind its field collimator) of 290 cm² and covers the energy band 2-18 keV in 8 channels. The channel boundaries are marked in Fig. 1, which shows sample pulse height spectra taken as the source intensified. These raw data have not been corrected for the detector efficiency and at this stage include no input spectral assumptions. Two points emerge clearly: when first detected the X-ray spectrum of A0620-00 was hard and remained so as the overall intensity rose to the precursor peak and, secondly, the subsequent rise to maximum intensity was entirely due to a strong enhancement of X rays below 10 keV, giving a marked softening in the overall spectrum. In fact, the observed flux above 10 keV actually fell during this period.

To quantify these changes, the pulse height spectra have been compared with a range of input spectra defined by a spectral parameter (power law index or thermal spectrum kT) and cutoff energy, folded with the known detector response and normalised to the observed data. Table 1 summarises the best fits obtained for spectra at different times in the X-ray light curve, χ^2 indicating the quality of a particular fit. The notable softening of the spectrum as the source brightens can now be quantified as, for example, an increase in the power law index from 0.6-0.7 in the precursor peak to ~ 4.0 near maximum brightness. The corresponding thermal spectral fits show a corresponding decrease in kT from ~ 30 to 1.5 keV. The rather large values of χ^2 indicate that the spectrum is generally too complex to be fully described by a single parameter fit, most spectra having excess observed counts in the upper one or two energy channels.

Figure 2 shows the input power law spectrum which matches the data quite well from 2 to 15 keV for modified Julian date (MJD) 40.5, just after maximum brightness. Also shown are two higher energy points obtained over the period MJD 44.5-47.5 with the Imperial College scintillation counter on Ariel V (A. Engel, personal communication), during which time the intensity remained steady. Again, the highest energy SSE spectral point shows some excess counts, and the presence of a harder component above 15 keV is supported by the Imperial College data. The best fit power law of Fig. 2 includes

Table 1 Parameters of best-fit X-ray spectra

Modified Julian date(42,600+)	Power law input spectrum				Thermal input spectrum			
	$I(E) = AE^{-\alpha} \exp(-E_a/E)^{8/3} (\text{keV cm}^{-2} \text{s}^{-1} \text{keV}^{-1})$				$I(E) = \frac{A \exp(-E/kT)}{E^{0.3}} \exp(-E_a/E)^{8/3} (\text{keV cm}^{-2} \text{s}^{-1} \text{keV}^{-1})$			
	$\alpha(\pm 2\sigma)$	$E_a \pm 2\sigma$ (or 2σ upper limit)	A	$\chi^2_{\min}$	$kT(\text{keV})$	$E_a(\text{keV})$	A	$\chi^2_{\min}$
28.8–29.4	0.65 ± 0.15	< 1.0	5.5 ± 0.6	56	20^{+15}_{-5}	< 0.7	3.7 ± 0.4	83
30.1–30.5	0.6 ± 0.15	< 1.3	13.2 ± 2.8	27	29^{+10}_{-15}	< 1.0	9.9 ± 1.6	38
31.0–31.4								
(a) $E < 6.2 \text{ keV}$	1.4 ± 0.1	< 1.0	71	12	8.7	< 0.5	22	280
(b) $E > 6.2 \text{ keV}$	0.2 ± 3.0							
32.72	3.1 ± 0.2	< 1.2	$(1.83 \pm 0.47)10^3$	31	1.36 ± 0.1	< 0.75	950 ± 110	152
33.14	3.7 ± 0.25	1.85 ± 0.25	$(7.1 \pm 2.3)10^3$	19	1.35 ± 0.07	< 0.8	$(1.18 \pm 0.17)10^3$	78
34.20	3.75 ± 0.15	1.9 ± 0.15	$(11.9 \pm 3.3)10^3$	27	1.37 ± 0.07	< 0.6	$(1.4 \pm 0.2)10^3$	100
35.10	4.0 ± 0.2	2.15 ± 0.15	$(8.8 \pm 2.6)10^3$	25	1.36 ± 0.05	< 0.75	$(6.1 \pm 0.5)10^3$	74
40.5	4.15 ± 0.2	2.6 ± 0.2	$(20 \pm 7)10^3$	22	1.52 ± 0.1	$1.1^{+0.4}_{-1.1}$	$(1.05 \pm 0.3)10^3$	26

a finite low energy cutoff of $E_a = 2.6 \text{ keV}$ and examination of all the existing spectra suggests a significant low energy attenuation becoming evident as the source intensifies.

The spectral evolution reported above provides interesting insight into the nature of A0620–00. Considering a binary model with unstable mass transfer, as referred to briefly in the preceding letter, the hard ‘precursor’ emission may be interpreted as arising from the hottest regions of an accretion disk lying close to the compact star. Then, as further mass is accreted, the disk grows rapidly, giving a corresponding increase in the overall X-ray intensity. Since the outer parts of this enlarged disk will be cooler the resulting X-ray spectrum will soften. Furthermore, the reduction in flux above 10 keV as the source increases towards maximum brightness may well be due to the hot inner regions of the disk being partially

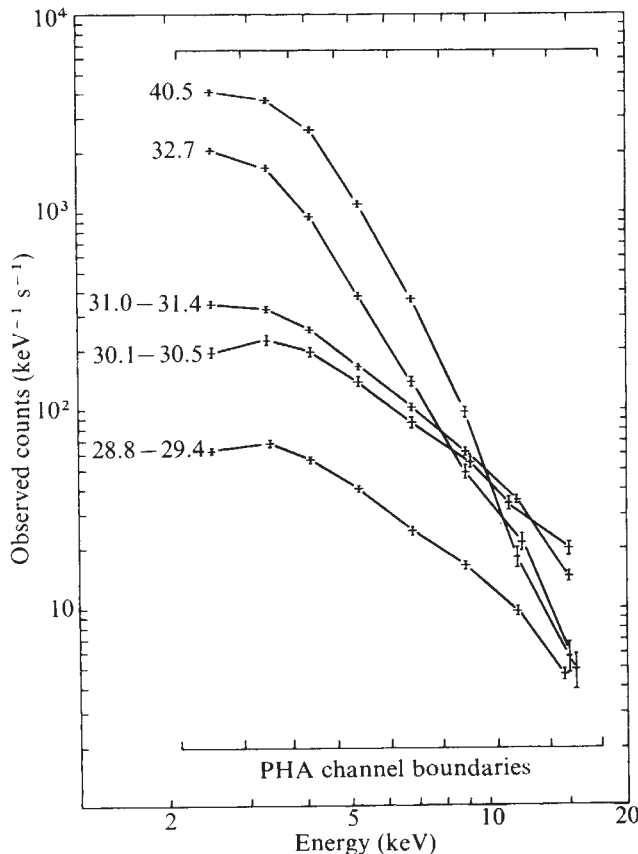


Fig. 1 Pulse height spectra of A0620–00 obtained during the rise to maximum brightness. The time of each spectrum is marked in MJD and error bars are $\pm 1\sigma$ for each spectral channel.

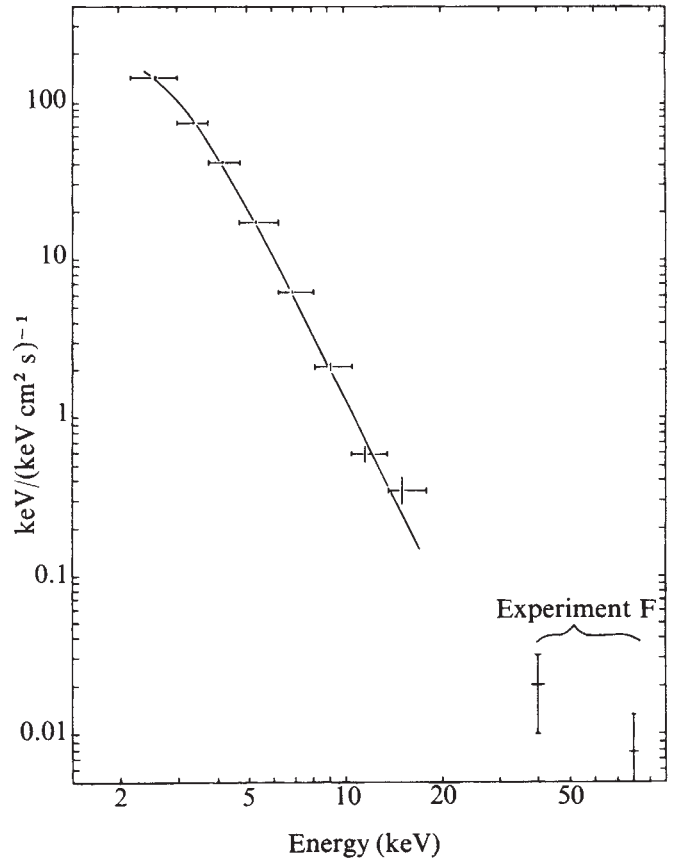


Fig. 2 The best-fit power law spectrum for the SSE data of MJD 40.5 (spectral index 4.1, low energy cutoff 2.6 keV) together with higher energy points from the Imperial College crystal spectrometer (experiment F on Ariel V) taken during MJD 44.5–47.5. Error bars are $\pm 1\sigma$.

obscured by the mass of outlying gas. A detailed analysis of the light curve and spectral evolution of A0620–00 presented here may be anticipated to yield important details on the accretion flow and disk formation in such close binary systems.

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Observations of A0620-00 at 962 and 151 MHz

FOLLOWING the discovery by the X-ray astronomy group at Leicester University of a large increase in the X-ray flux of A0620-00, observations at 962 MHz and 151 MHz have been made at Jodrell Bank. During the period August 16-29, observations were made at 962 MHz with the MK II-MK III long baseline radio-link interferometer. This instrument has a spacing of 23.7 km giving a lobe size of $\sim 3''$ at this frequency. The radio-link is phase compensated so that integrations can be made for several hours.

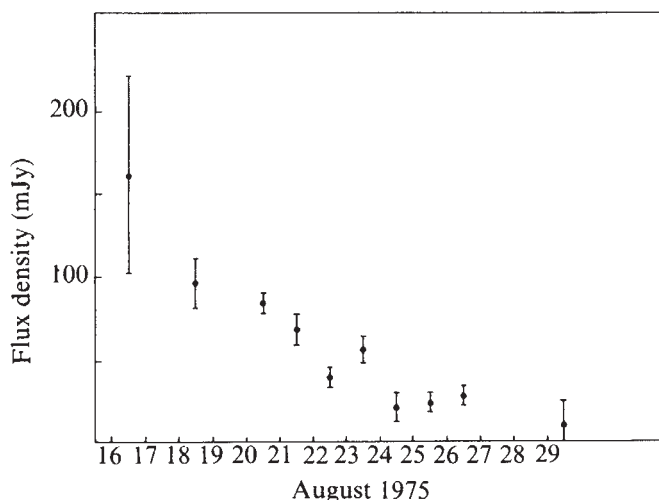


Fig. 1 Flux density of A0620-00 at 962 MHz with a resolution of $3''$

On August 16 and 18, the instrument was used to survey an area 0.5° by 0.5° centred on the position of the X-ray source¹. For a source at declination 0° the synthesised beam after integrating from hour angle 1.5 – 3.5 h is $5''$ in right ascension and $30''$ in declination. In the area surveyed only one source, at the extreme edge of the field, was significantly above the noise level. On August 19 the position of an optically variable object, possibly associated with the X-ray source, was communicated to us². The right ascension of this object and the observed radio source agreed to within $6''$ making an association likely. Further observations were therefore restricted to a smaller area of sky surrounding the position of the radio source and optical object. Thus a wider bandwidth could be used, giving an increased sensitivity and enabling the position to be measured with greater accuracy. With the 4-MHz bandwidth the r.m.s. noise on an amplitude measurement after a 2-h integration was 6 mJy. The position measured on August 18 was: $\alpha = 06$ h 20 min 11.4 $\pm$ 0.27 s; $\delta = -0^\circ 17' \pm 7''$ (1950.0). Subsequently, more accurate measurements of the X-ray source position confirmed the radio identification^{3,4}. No significant variation of fringe amplitude with hour angle was detected during the observations, thus indicating that the angular size of the radio source was less than $3''$. Measurements of the flux density of the radio source were continued until August 29 and showed a decreasing intensity with time as shown in Fig. 1.

During August 12-15 observations were also made at 962 MHz using the MK II radio telescope. Total power measurements indicated that the flux density of the source was less than 5 Jy. On August 17, 20, 23 and 24 observations were made using the MK IA-Defford radio-link interferometer at 151 MHz. This instrument has a baseline of 127 km which gives a lobe size of $3''$ at this frequency. No radio emission was observed down to a sensitivity limit of 250 mJy. At this frequency, however, interstellar scattering may increase the apparent angular

size of a source near the galactic plane, as in the case of Cyg X-3 (ref. 5), and this could explain why no source was observed in the present observations at 151 MHz.

If the decrease of flux density shown in Fig. 1 is expressed as an exponential decay, the time for the flux density to fall by a factor of $1/e$ is 5.2 d. The data may also be fitted to a power law of the form $t^{-3 \pm 1}$ on the assumption that the outburst originated on August 3.0 UT. On the simple van der Laan model⁶ of uniform expansion of an optically thin cloud of relativistic electrons, this would correspond to a radio spectral index, α , of -0.25 ± 0.25 where α is defined by $S = S_0 \nu^\alpha$.

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The optical counterpart of A0620-00 before its eruption

AN accurate position of the optical counterpart of A0620-00 discovered by Boley and Wolfson¹ has made possible the identification of the image of the star on the Palomar Observatory Sky Survey (POSS) charts.

The position was measured on two 10-min exposures on Eastman Kodak Ila0 plates taken on the 13-inch astrographic telescope at Herstmonceux on August 27-28 and 28-29, 1975 relative to standard positions of AGK3 stars, using a Zeiss x - y measuring engine. It was reduced using the methods described by Murray, Tucker and Clements². The mean position of the Boley-Wolfson star which appears at $B \sim 12.2$ on both plates is: $\alpha = 06$ h 20 min 11.176 $\pm$ 0.021 s; $\delta = -00^\circ 19' 10.80 \pm 0.32''$ (1950.0), where r.m.s. errors are quoted. There is a substantial contribution to these errors from the uncertainty of the system defined by the AGK3 stars and from the possible effects of refraction if the identification has a different colour from the AGK3 stars. The right ascension lies about 3 s.d. ($1.20 \pm 0.35''$) from that of the radio object measured at the Mullard Radio Astronomy Observatory³. The position is, however, within the X-ray error boxes measured by the Leicester⁴ and Birmingham-Mullard Space Science Laboratory⁵ experiments on Ariel V and by the Massachusetts Institute of Technology experiment⁶ on SAS-3.

The positions of a number of stars on the POSS charts (from originals taken on November 18-19 1955) were also determined with respect to the same AGK3 standards from measurements made with a Faul Coradi Coradigraph x - y machine. Similar reductions showed that one star, identified in Fig. 1, lies at: $\alpha = 06$ h 20 min 11.202 $\pm$ 0.023 s; $\delta = -00^\circ 19' 10.45 \pm 0.35''$ (1950.0).

In comparing these two positions the errors of the positional system defined by the AGK3 stars cancel and so the difference in position has smaller errors than the appropriate combination of the errors in the positions. The differences between the measurements obtained on the POSS and the 13-inch plates are: $\Delta\alpha = +0.39 \pm 0.38''$ and $\Delta\delta = +0.35 \pm 0.38''$. Since the positions agree very well and the next nearest object on the POSS is $\sim 15''$ away, the star identified in Fig. 1 is very likely to be the optical counterpart before its eruption.

Eye estimates on the POSS give magnitudes in 1955 for the counterpart of $B \sim 20.5$, $B - R \sim 3.6$ where the errors are likely

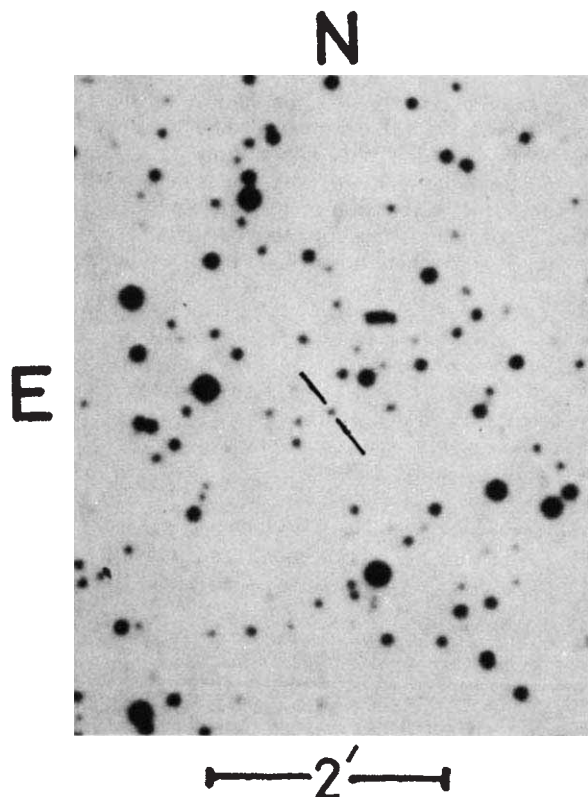


Fig. 1 Chart identifying the image of the optical counterpart of A0620-00, before its eruption, on the POSS red chart (copyright: National Geographic Magazine-Palomar Observatory Sky Survey).

to be ~ 0.5 mag. From these magnitudes the star is unlikely to be a giant since it would then lie at a distance of at least 15 kpc which would place it far from the plane and pose difficulties in reconciling the observed X-ray flux with the Eddington limit⁴ if the X-ray source has a mass similar to that of the Sun. If it is a K dwarf the estimated colour would demand some obscuration, presumably circumstellar perhaps arising from earlier outbursts⁷, and a distance of the order of 500 pc. Such obscuration would be of interest in view of reports⁸ of interstellar lines. In addition, the magnitude expected⁴ from extrapolating the X-ray spectrum near maximum to the optical region as an optically thin bremsstrahlung source, is $B \sim 8$, which may also indicate the presence of interstellar absorption, although it could also represent self-absorption in the hot gas emitting the X-rays.

If the optical counterpart before eruption is a red dwarf star, this is consistent with a model for the X-ray source resembling current explanations of dwarf novae (see for example ref. 9), but with a black hole or neutron star replacing the white dwarf as the body accreting material spilt from the Roche lobe of the red star.

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Galactic superhaloes, globular clusters and high latitude X-ray sources

I show here that the observational features of unidentified, high latitude, X-ray sources¹ are consistent with the suggestion that they are a population of remote, optically undetectable, globular clusters. The principal objection to the hypothesis that the high latitude sources are members of any galactic population is their isotropy in galactic longitude. But if it is assumed that the most distant unidentified high latitude source radiates at 10^{38} erg s⁻¹ (a typical value for a bright globular cluster X-ray source^{2,3}) and that it is the faintest unidentified high latitude source, visible at 1-2 Uhuru counts s⁻¹, its distance would be ~ 200 kpc. Then, extrapolating to the brightest unidentified source at 7 counts s⁻¹, we get a lower bound on the minimum distance to any objects of about 80 kpc, and the volume toward the galactic centre, between $-90^\circ < l^{\text{II}} < 90^\circ$ in the 'shell' from 80 to 200 kpc, differs negligibly from the volume toward the anticentre, at $90^\circ < l^{\text{II}} < 270^\circ$. Although a specific radiation model is used in this case, the luminosities do not affect the basic result.

It is widely believed that the globular cluster population of the Galaxy is essentially completely observable⁴; however, that may not be the case. Table 1 shows concentration class

Table 1 Average absolute photographic magnitude of different classes of globular clusters*

Class	M_{pg}	Class	M_{pg}
I	-7.9	VII	-7.5
II	-7.8	VIII	-7.2
III	-7.8	IX	-6.7
IV	-7.8	X	-6.2
V	-7.7	XI	-5.7
VI	-7.6	XII	-5.2

*Data from ref. 5.

against average absolute photographic magnitude for 91 clusters⁵, and it can be seen that as the cluster gets more diffuse (higher number class), the integrated absolute magnitude falls. Indeed, out to an apparent value of $m_{pg} = 15$, all clusters are known only to concentration class X or tighter⁴. And, in fact, 10 of the 12 'new' globular clusters detected in the Palomar Sky Survey plates are in class XI or XII (ref. 6). These new clusters are difficult to detect because of three characteristics: they are intrinsically fainter than the others, they are diffuse (and thus more difficult to discriminate from the general field), and they are far away.

Pal 3 and Pal 4 have been examined photometrically⁷. Those particular clusters are about 100 kpc away, and it is possible that because of their faint absolute magnitude, such objects may populate space in great numbers and remain undetected⁷. The average B magnitude of the 25 brightest stars in these clusters is ~ 20 ⁴; even Pal 12, 33 kpc away, has an average B magnitude of 17.5 for its 25 brightest stars. These faint magnitudes, and the diffuse nature of the clusters make it likely that the NGC and IC catalogues would not include other similar objects⁴. Since the limiting red magnitudes of the Palomar plates are ~ 20 ,—assuming that the red giant stars have $M_v = 0.0$ with the total integrated cluster magnitude $M_v = -6$ (ref. 7; see also Table 1)—then all clusters fainter than $m_v \approx 14$ will probably be undetectable. Pal 3 and Pal 4 have m_v values of 14.2 and 14.4, respectively⁷.

The difficulty of detecting distant faint clusters is demonstrated by Fig. 1. Clearly, the visible globular clusters are on the edge of detectability.

The distribution of the number density of the globular clusters against the distance from the galactic centre^{4,8} describes well the spatial density of the nearer clusters, but that is not so at large distances. Figure 2 shows the number of globular clusters in 10 kpc shells, against the distance from the galactic centre, for all clusters whose distances are known¹.

Further, 6 globular clusters have been observed at > 80 kpc and none at all have been observed between 50 and 80 kpc. To explain these observations, I propose that the density distribution of globular clusters changes, becoming roughly proportional to the volume element at a point in space 50–80 kpc from the galactic centre. With such a model a preponderance of clusters could be expected at about the limit of detectability, since beyond that point the clusters would not be visible by definition, whereas at closer distances the volume surveyed and thus the number of objects observed would be smaller.

Approximately 150 unidentified, high latitude, X-ray sources have been observed down to ~ 1.5 Uhuru counts s^{-1} (after coverage corrections)¹. If it is assumed that X-ray sources occur in these remote, diffuse clusters in the same ratio—4/119—as in the nearer clusters², about 4,500 class XI or XII globular clusters would have to be present between roughly 80 kpc, where the first 'distant' clusters are observed, and the limit of 200 kpc. Assuming a constant space density gives: $\rho = 1.3 \times 10^{-4}$ clusters kpc^{-3} . That is almost exactly equal to the space density of globular clusters between 20 and 30 kpc; and 4,500 clusters with an initial mass of $\sim 10^5 M_{\odot}$ are still much less massive than the Galaxy.

The possibility that high velocity stars form 'moving groups', belonging to the halo population, has been discussed^{9,10}, and it has been suggested that these groups may share an equality of motion because they are remnants of globular clusters. It has been estimated that the number of parent globular clusters is of the order of 10^3 (ref. 10).

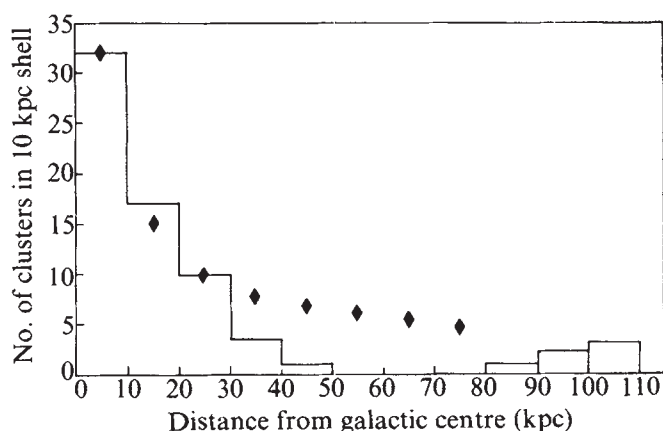


Fig. 2 Number of globular clusters in 10 kpc shells against distance from the galactic centre. $\blacklozenge$, Predicted values based on $\rho \propto r^{-2.7}$ normalised to the value observed between 0 and 10 kpc; χ^2 per degree of freedom is 0.8 out to 40 kpc, and 3.3 out to 80 kpc. For a $\rho \propto r^{-2.9}$, χ^2 is 1.4 and 2.4 out to 40 kpc and 80 kpc, respectively.

The observational implications are clear: searches should be made for variability, and the positional accuracy of the high latitude X-ray sources should be improved. Also, deep sky plates in the red should be made. Further distance estimates to clusters for which information does not already exist would help in understanding the true density distribution function. If, indeed, the unidentified high latitude sources are stellar objects in a superhalo population of diffuse globular clusters, then the log N -log S curve¹ should flatten beyond the true edge of the halo.

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Gas well pressure fluctuations and earthquakes

AN important part of earthquake prediction studies is the search for various precursors. It is likely that one precursor may not enable us to predict an earthquake correctly, but a collection of these may remove the uncertainties. It is also desirable to find data that are recorded routinely, in enough detail, such that they can be conveniently used by seismologists.

In so far as the permeability (due to pores and/or joints) and the pressure gradient in a reservoir may be influenced by stress, production and well-head pressure data from oil and gas wells may prove to be useful. There has been an attempt to search for precursory phenomenon in monthly oil well production data¹. On the other hand, many examples exist already of changes of levels and flow rates in water wells before and after earthquakes^{2,3}; it is reasonable to expect that oil and gas wells would behave in much the same way.

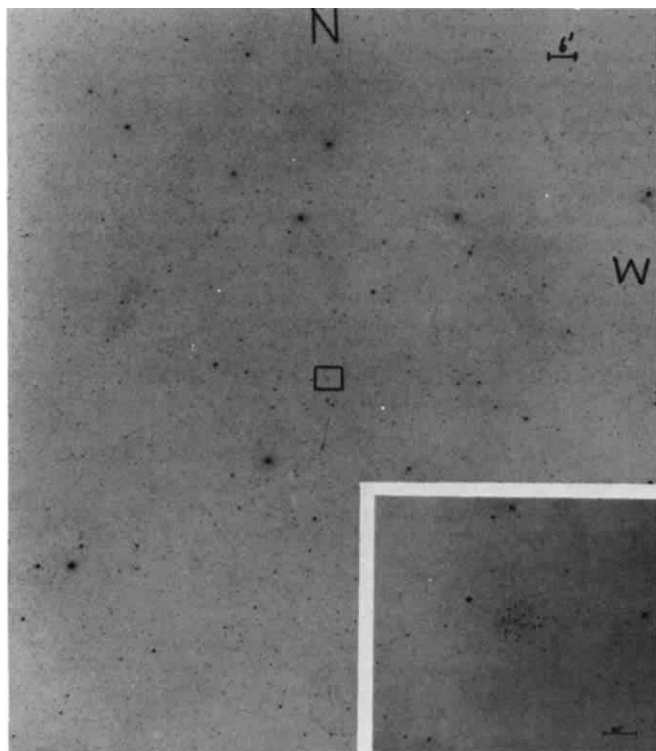


Fig. 1 Region around Pal 4, a remote globular cluster. Inset, 200'' plate of the same object taken by Burbidge and Sandage⁷ (covering the area of sky boxed in the main figure). Scale bar, 6'; inset scale bar, 60''.

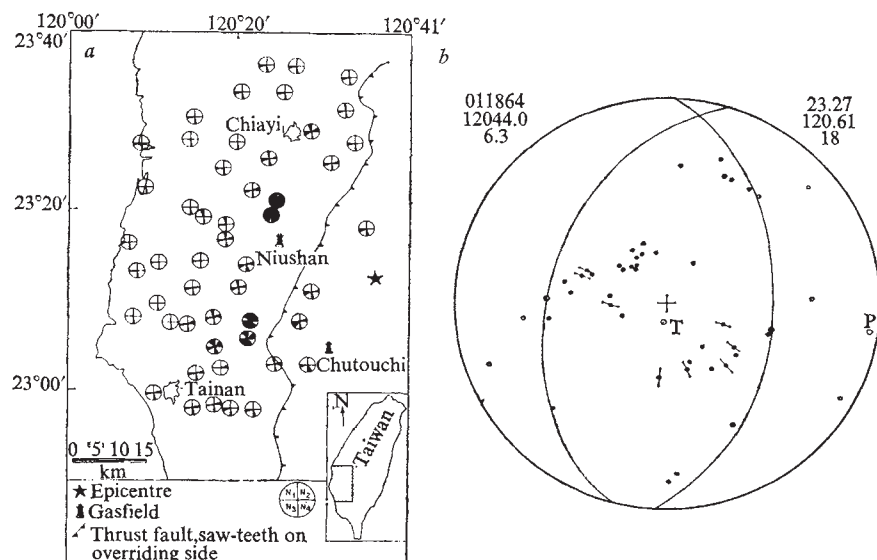


Fig. 1 *a*, The location of the earthquake epicentre, the causative fault and the Niushan and Chutouchi gas fields. The damage figures are indicative of the extent of the source region to the west of the fault, where the towns (indicated by circles) are of approximately equal size in terms of population and number of structures. To the east of the fault, in the mountainous area urbanisation is much less dense. The numbers $N_1, N_2, \dots$ are normalised to the maxima. N_1 , no. of dead; N_2 , no. of wounded; N_3 , no. of houses collapsed; N_4 , no. of houses damaged. *b*, The lower hemisphere equal area projection of the focal mechanism of the January 18, 1964 earthquake. T, tension axis; P, compression axis.

Here we report a case of pressure or production changes in two gas fields in Taiwan before a 6.75 magnitude (Pasadena M_s) earthquake.

Figure 1a shows the location of the Niushan and Chutouchi gas fields, the epicentre and the extent of damage. This region is very active seismically and since the mid-1600s, when historical records became available in this area, many destructive earthquakes have occurred here. The earthquake of January 18, 1964 (origin time 1204:40.0 GMT; depth 17 km (W. H. K. Lee and J. P. Yang, unpublished)) is apparently related to an east dipping thrust fault mapped by surface as well as subsurface geological data. The fault plane solution is shown in Fig. 1b. From the magnitude of the event and the damage pattern (Fig. 1a), the fault length associated with this earthquake is of the order of 15–35 km (ref. 4); the Niushan field is within 8 km and Chutouchi field 12 km of the surface trace of the causative fault and are therefore within the source region of the earthquake.

Niushan gas field produces from upper Pliocene units that are composed of alternating beds of sandy shale, silty sandstone, shale and fine to medium grained sandstone. Chutouchi field produces from lower Miocene fine compact sandstone and badly sorted silty sandstone. In both fields the porosity is low and the gas was produced from jointed and fractured rocks⁵.

Both gas fields were in operation between 1957 and 1970. Of the more than ten wells in Niushan gas field, only three were not pumped. Ideally both well-head pressure and production rate data for such wells are useful, since both are related to the change in permeability and/or pressure gradient in the reservoir resulting from tectonic stress. The production data in Niushan field are not usable, however; apparently the total production of the field, including pumped wells, is correct, but the individual readings were rough estimates.

Because of the presence of water in the well and the bubbling of gas, the pressure varies between daily maxima and minima; the pressure is recorded continuously, but only the maxima and minima were entered in the production records which were used in this work. In Fig. 2, the daily maxima and minima since January 1, 1964 are plotted for the three wells. There are rather clear increases (15–60%) in pressure for all three wells for both daily highs and lows, starting from January 8. Careful examination of records covering several years shows that such rapid change did not appear; the pressure readings have short term variations of $\pm 0.1 \text{ kg cm}^{-2}$ superposed on a very slowly decreasing trend for several years before the earthquake. After the earthquake, there was a noticeable short term fluctuation in one of the wells, but not in the other. It should be noted that a break in the piping system occurred during the earthquake resulting in open flow of the wells and an ensuing drop in well

pressure. The pressure returned to the level existing before January 9 after the pipes were repaired on January 20, although one of the wells (R-10) was permanently damaged during the earthquake.

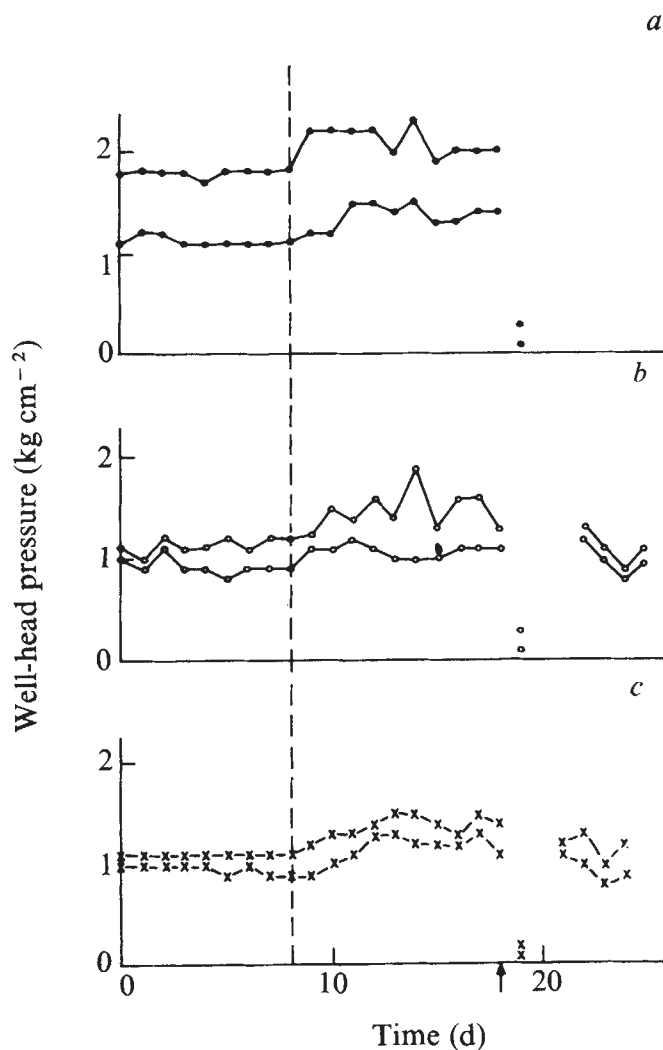


Fig. 2 The well-head pressure variation before and after the January 18, 1964 earthquake (arrow) at Niushan gas field. *a*, Well R-10, depth 988.6 m; *b*, well R-7, depth 717 m; *c*, well R-23, depth 385 m.

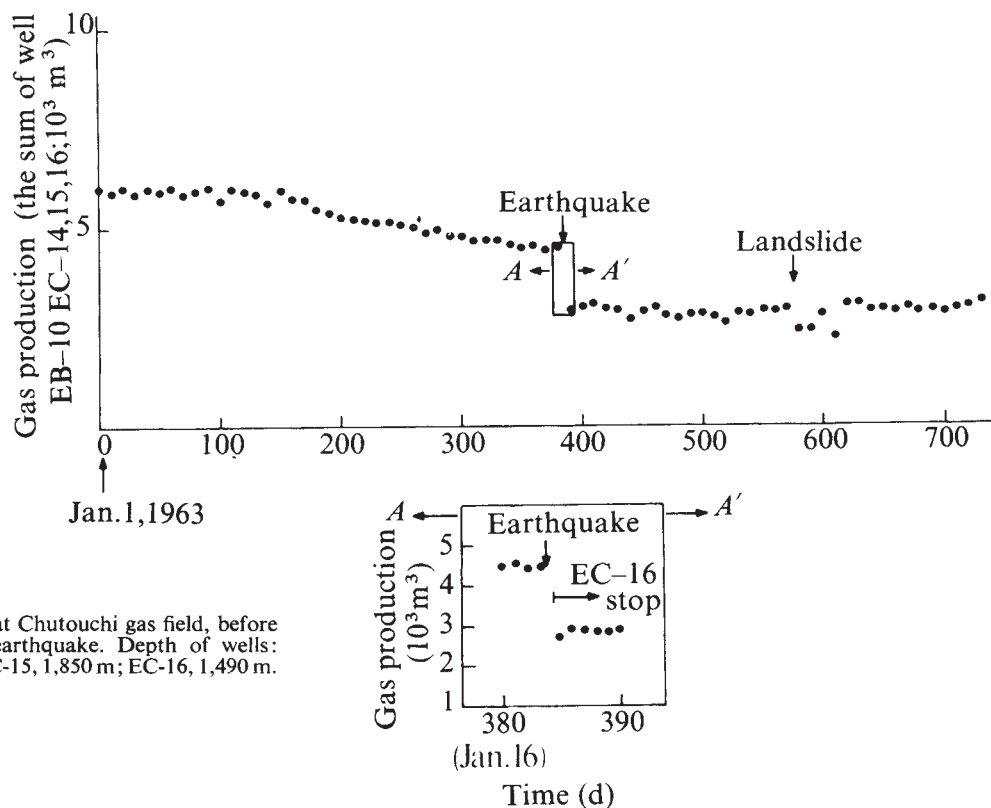


Fig. 3 Gas production rate data at Chutouchi gas field, before and after the January 18, 1964 earthquake. Depth of wells: EB-10, 1,440 m; EC-14, 1,324 m; EC-15, 1,850 m; EC-16, 1,490 m.

The increase in pressure before the January 18 earthquake is apparently a precursor to the earthquake.

At the Chutouchi gas field, on the hanging wall side of the thrust (Fig. 1), the pressure and production rate showed only slight changes during the period of January 1–18. We then looked at the production rate data for all three wells from 1958 and found that superposed on a general long term slow decrease, there is a noticeably more rapid drop beginning after March 1963 (Fig. 3); after the earthquake the trend became steady again. (The drop in total production after the earthquake was caused by the disruption of one well during the earthquake.)

The difference in well pressure behaviour at these two fields before the earthquake may indicate the different behaviour of reservoirs under stress on two sides of the causative fault; Niushan gas field is on the foot wall side and Chutouchi field on the hanging wall side. The exact mechanism of this behaviour can be postulated only after the stress field around the fault and the joint system are known in detail.

As demonstrated by Myachkin and Zubkov⁶, earthquake precursors can be classified into short term and long term. The Niushan pressure change can be classified as a short term precursor, although the time interval between the commencement of this precursory change and the occurrence of the earthquake is an order of magnitude longer than most of the results of Myachkin and Zubkov (see figures in ref. 6). On the other hand, if the Chutouchi data are a true precursor, then they are closer to being a long term precursor.

Because the pressure and production data are continuously monitored by the oil companies, they may be a worthwhile source of evidence of the existence and usefulness of this precursor in other gas and oil fields.

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Gravitational drive on oceanic plates caused by thermal contraction

PROPOSED mechanisms for the motion of lithospheric plates over the Earth's surface range from the self-drive of plates by gravitational sliding^{1,2}, through passive response to mantle-wide convection³, to point drive by upwelling hotspots in the mantle⁴. All of these models depend on the distribution of effective viscosity within the mantle. Investigations⁵ have established the strong likelihood that a weak layer (10^{20} poise) exists immediately below the lithosphere, and long spatial-wavelength loadings associated with changes in the Earth's rotation⁶, together with the larger scale components of glacial loading⁵, demand that this asthenosphere be quite thin (100 km) and bounded by material from the lower mantle with a viscosity of two to four orders of magnitude higher.

As an analogy, this situation can be visualised as a layer of ice, over a layer of water, over a layer of honey 30 times as deep. Thus it can be seen that the plates (the ice in this analogy) would not be well coupled to motions in the lower mantle (the honey), and, indeed, that the highly viscous lower layer would be unlikely to flow faster than the surface layer. Several observations support this concept. Morgan⁷ has constructed plate histories that require very little relative motion between hotspots, and there is significant coincidence between the hotspot frame of reference and a frame of reference derived by minimising the motion of plate boundaries⁸.

The boundary-layer theory of plate accretion has received strong support from the discovery of the square-root law of

ridge topography⁹, from which the depth of the oceanic basement is shown to be proportional to the square root of its age between 0.5 and 80 Myr. This would be expected if it were due solely to the thermal contraction of uniformly flowing material. Here I recalculate the driving force consequent on the ridge topography, in terms of a thermal boundary layer model.

The method used is similar to those used by Frank² and by Artyushkov¹⁰, except that a continuous density variation is retained and handled by the similarity rule for the slope of the temperature curve in a thickening boundary layer. My calculations consider an increment, Δx , of lithosphere with surface slope, α , bottom slope, β , and thickness, l (Fig. 1). The origin of z is set at the initial height of the lithosphere surface where the pressure is P_0 , and the forces within the lithosphere are simplified to the lithostatic pressure, P , and a horizontal deviatoric compression, σ . Vertical and horizontal shear stresses, and the overall bending moment, can be neglected because horizontal motion is controlled by the pressure forces, the summed horizontal force, F , and the viscous stress, τ , on the base of the segment. Variations in the third dimension are ignored because the spreading is uniform along a given segment of ridge crest. The asthenosphere is assigned a density, ρ_A , and the lithosphere is denser than this by an amount $\rho(x, z)$. The x dependence of the density of the lithosphere will be handled by using a thermal definition of the base of the lithosphere and the similarly law for thermal diffusion solutions. A short distance from a spreading ridge-crest the temperature distribution in an upper mantle of constant diffusivity, κ , becomes indistinguishable¹¹ from

$$T = T_1 \operatorname{erf}(z/2\sqrt{\kappa t}) \quad (1)$$

and the definition of a lower lithosphere boundary depends on the choice of an arbitrary ratio $T/T_1 = \operatorname{erf} y$, at which the material can be considered to have become rigid. Even in the case of varying parameters, the form of $\rho(z)$ will remain unchanged provided only that a depth sensitive phase change be absent⁹. The lithostatic pressures can therefore be expressed:

$$P_1 = \int_0^z g [\rho_A + \rho(x_1, z)] dz + P_0 \quad (2)$$

$$P_2 = \int_0^z g [\rho_A + \rho(x_2, z)] dz + \alpha \Delta x \rho_w g + P_0$$

but

$$\rho(x_2, z) = \rho \left[x_1, \frac{l(z - \alpha \Delta x)}{l + \beta \Delta x - \alpha \Delta x} \right] = \rho(x_1, z')$$

so

$$P_2 = \int_0^{\frac{z - \alpha \Delta x}{1 + \theta}} g [\rho_A + \rho(x_1, z')] (1 + \theta) dz' + \alpha \Delta x g \rho_w + P_0$$

$$= (1 + \theta) P_1 \left(\frac{z - \alpha \Delta x}{1 + \theta} \right) + \alpha \Delta x g \rho_w - \theta P_0$$

where $\theta = \Delta x(\beta - \alpha)/l$

The total pressure forces acting on the vertical sides of the segment are found by integration, so that by a similar variable transformation:

$$\int_{\alpha \Delta x}^{l + \beta \Delta x} P(x_2, z) dz = (1 + \theta) \int_0^l P(x_1, z') dz' + \alpha \Delta x g \rho_w (1 + \theta) - \theta P_0 (1 + \theta) \quad (3)$$

The full, horizontal force balance-equation can now be written

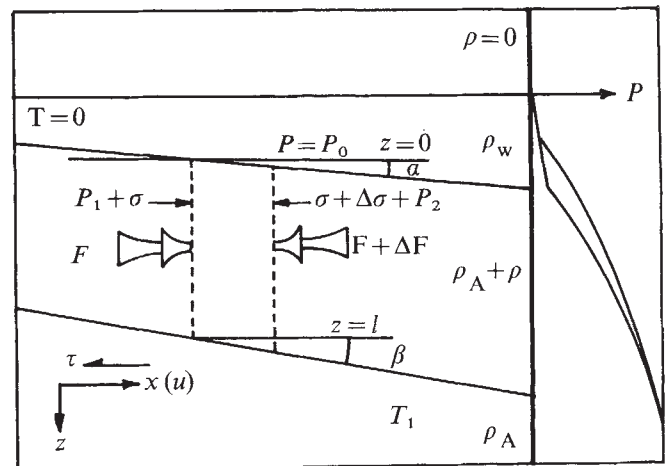


Fig. 1 Schematic diagram of the principal forces on a segment of lithosphere ($\rho_A + \rho$), underlying ocean (ρ_w) and resting on a uniform asthenosphere (ρ_A). The slopes of top and bottom are considered linear since the segment thickness Δx will be taken down to zero during the analysis. The inset (right) compares the hydrostatic pressure profiles at the two edges of the diagram, assuming isostatic compensation. Density variations are greatly exaggerated.

out, including the terms consequent upon the horizontally resolved pressures on the top and bottom:

$$\Delta F - \tau \Delta x = \int_0^l P_1 dz - (P_0 + \frac{1}{2} \rho_w g \alpha \Delta x) \alpha \Delta x - \int_{\alpha \Delta x}^{l(1+\theta)} P_2 dz + [P(x_1, l) + \frac{1}{2} \rho_A g \beta \Delta x] \beta \Delta x$$

Anticipating the division through by Δx and letting $\Delta x \rightarrow 0$, all terms containing Δx^2 can be neglected, so that this equation simplifies, using equations 2 and 3, to:

$$\Delta F - \tau \Delta x = \beta \Delta x \int_0^l g [\rho_A + \rho(x_1, z)] dz - \alpha \Delta x g \rho_w l - 2\theta \int_0^l \int_0^z g [\rho_A + \rho(x_1, z)] dz dz$$

$$dF/dx - \tau = \alpha g (\rho_A - \rho_w) + \beta g \int_0^l \rho(z) dz - (2(\beta - \alpha)g/l) \int_0^l \int_0^z \rho(z) dz dz \quad (4)$$

expressing the drive resulting from the surface slope and from internal density differences. For the trivial case of $\rho(x) = \rho'$, constant through the lithosphere, the result is simply $\alpha g (\rho_A + \rho' - \rho_w)$, and it should be noted that this result does not agree with that of Jacoby¹. For linear slopes, equation (4) can be integrated with respect to x readily, and a direct comparison can be made, for a lithosphere that vanishes at the ridge crest:

$$F - \int \tau dx = \frac{1}{2} h l g (\rho_A + \rho' - \rho_w) + \frac{1}{2} l^2 \rho g \quad (\text{from ref. 1})$$

where h is the drop in height from the ridge crest reference height (ideally, the origin of the square root topography line⁹). The result is in agreement with that of McKenzie¹², obtained by an unspecified method. The factors h and l , or α and β , are related in practice by the isostatic adjustment found to apply to oceanic crust from the very low free-air gravity anomalies.

A case of greater interest that is amenable to calculation is the cooling boundary layer lithosphere with a temperature distribution is given by equation (1). Constant diffusivity is

implied by this relationship, and the material must be assigned a constant volume expansion coefficient, γ , for the calculation of $\rho(z)$. Then

$$\rho = \rho_A \gamma (T_1 - T) = \rho_A \gamma T_1 \operatorname{erfc} [z/2\sqrt{(\kappa t)}]$$

giving h and, therefore, α (see ref. 9):

$$h = [1/(\rho_A - \rho_W)] \int_0^\infty \rho dz = (1/\sqrt{\pi}) [\rho_A \gamma T_1 2\sqrt{(\kappa t)} / (\rho_A - \rho_W)]$$

$$\alpha = \frac{dh}{dx} = \frac{1}{s} \frac{dh}{dt} = \frac{1}{\sqrt{\pi}} \frac{\rho_A \gamma T_1}{s(\rho_A - \rho_W)} \sqrt{(\kappa/t)}$$

where s was the spreading velocity of the lithosphere when it was formed, and $\beta - \alpha$ may be found from the slope of the locus, $l = 2y\sqrt{(\kappa t)}$ (constant y):

$$\beta - \alpha = \frac{dl}{dx} = \frac{1}{s} \frac{dl}{dt} = \frac{y}{s} \sqrt{(\kappa/t)}$$

thus, equation (4) becomes:

$$\begin{aligned} \frac{dF}{dx} - \tau = \frac{g\rho_A \gamma T_1 y \kappa}{s} \left[\frac{1}{\sqrt{\pi}} + \left(1 + \frac{\rho_A \gamma T_1}{y\sqrt{\pi}(\rho_A - \rho_W)} \right) \times \right. \\ \left. \times \left(\frac{1}{\sqrt{\pi}} (1 - e^{-y^2}) + y \operatorname{erfc} y \right) - 2 \left(\frac{1}{\sqrt{\pi}} (1 - \frac{1}{2} e^{-y^2}) - \frac{1}{4y} \operatorname{erfc} y + \frac{1}{2} y \operatorname{erfc} y \right) \right] \end{aligned}$$

where it should be noted that the integrals of the error function differ from the standard forms because of integration from 0 to y instead of from y to ∞ . Finally, this simplifies to:

$$\begin{aligned} \frac{dF}{dx} - \tau = \frac{g\rho_A \gamma T_1 \kappa}{s} \left[\operatorname{erfc} y + \frac{2\rho_A \gamma T_1}{\sqrt{\pi}(\rho_A - \rho_W)} \times \right. \\ \left. \times \left(\frac{1}{\sqrt{\pi}} (1 - e^{-y^2}) + y \operatorname{erfc} y \right) \right] \quad (5) \end{aligned}$$

the second term of which can be neglected for reasonable values of y , γ and T_1 . For example, ridge topography has been⁹ well fitted by using $\rho_A = 3.3 \text{ g cm}^{-3}$; $\gamma = 4 \times 10^{-5} \text{ }^\circ\text{C}^{-1}$; $\kappa = 8 \times 10^{-3} \text{ cm}^2 \text{ s}^{-1}$; $T_1 = 1,120 \text{ }^\circ\text{C}$ (Pacific Ocean). The parameter γT_1 is, therefore, 0.045, and the choice of y is not critical as is shown in Table 1.

Presuming that asthenosphere material becomes essentially rigid after a relatively small drop in temperature, the final result can be written simply as

$$dF/dx - \tau \approx g\rho_A \gamma T_1 \kappa / s = 7.2 \text{ bar} \quad (s = 5 \text{ cm yr}^{-1}) \quad (6)$$

the most startling feature of which is that it is independent of the age of the lithosphere. For comparison of figures, the drag on lithosphere moving at 10 cm yr^{-1} over 100 km of asthenosphere of 10^{20} poise, is 3 bar. In the absence of such drag, the lateral compressive stress developed over 5,000 km of plate 100 km thick at its end is $50 \times 7.2 = 360 \text{ bar}$.

The conclusions this analysis points to are both simple and important. The overall magnitude of the driving force is similar to that derived by some authors^{10,12} but not to that derived by Jacoby¹. There is no source for the high compressive stresses invoked by Watts and Talwani¹³ to explain the topographical bulge and high gravity on the seaward side of many trenches. The thermal contraction force is independent of the age of the lithosphere and is only dependent on the velocity at which it was originally spread. It is not an edge

Table 1 Variation of parameters with y

y	0	1	2	3
$T/T_1 = \operatorname{erfc} y$	0	0.843	0.995	0.99998
$h \propto \int_0^y \operatorname{erfc} y$	0	0.524	0.563	0.564

force associated only with active mid-oceanic ridge crests, as assumed by Forsyth and Uyeda¹⁴. This removes one of the two arguments advanced by McKenzie¹² against plate drive by ridges: that is, small plates should not necessarily move faster than large plates. Another argument¹² is that the mechanical work produced by the ridges is insufficient to account for the elastic energy released in earthquakes. This is easily refuted by the observation that the majority of energetic earthquakes are associated with subduction zones. More mechanical work is generated by sinking lithosphere than by gravitational sliding from ridges, but this work is not necessarily available to drive the plates. Analysis based on the correlation of plate movement with boundary composition¹⁴ shows that the subduction zones contain both the largest driving force and the greatest resistance to flow. Although only edge drive by ridges is permitted, the conclusion arises inexorably from the fact that all fast moving plates are bounded by trenches. Unfortunately, this may merely reflect the inability of any fast-moving plate to override another without generating a huge resistance.

The thermal contraction drive is ample to push plates over any asthenosphere that complies reasonably with the known constraints, and the presence of subduction zones may exercise indirect control over plate motions by providing the only available low resistance against the forward end for fast moving plates. A more serious objection to drive by thermal contraction is the fact that in large mountain ranges the lateral stresses in the lithosphere would have to be about three times greater than can be provided by ridges^{2,10}. The answer to this objection is, in part, similar to that to the problem of earthquake energy: the largest mountain ranges are associated with sinking slabs, present or past. The excess hydrostatic pressure at shallow depth under an isostatically compensated mountain range can be partially balanced by a pressure deficit at intermediate depth if the region is underlain by anomalously dense mantle. An extra oceanic slab, slung flat beneath a mountain range, can provide a regional 'suction' equal to the drive provided by the ridge, and that is increased further in the extra mountain 'root' region needed to compensate for the slab. Long tilted slabs can create even larger 'suction' forces. The other class of substantial mountain ranges, associated with continental rift zones, are probably uplifted by thermal expansion and magma buoyancy¹⁰. Since they are rifting, compensatory horizontal compression is not required.

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Coated optical guides for spectrophotometry of chemical reactions

A NEED exists for a method of detecting small amounts of chemicals in air and other gases, and in solutions, simply, and with a high degree of sensitivity. We have developed a new technique based on the concept of using waveguide phenomena in combination with chemical reactions. This technique is applicable to a wide variety of chemical reactions, including biochemical systems, provided that they cause a change in light transmittance by virtue of a change in colour, refractive index, or light scattering. Both real-time and cumulative measurements are possible.

The theory underlying light guide phenomena is well understood and has been treated extensively by Kapany^{1,2}. We felt it should be possible to alter the transmission properties of an optical guide using a chemical reaction on the surface of the guide, since a change in refractive index at the interface with the rod surface alters the critical angle. Initially, reactants were applied directly to a rod which functioned as a lightguide. Changes in light intensity were observed qualitatively as a

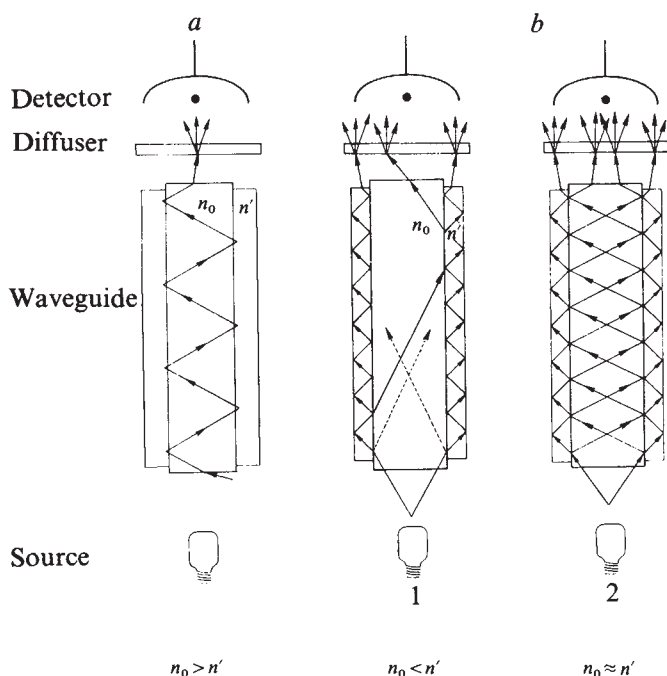


Fig. 1 Mechanism models.

reaction was carried out on the surface. But problems in coating adhesion were encountered using this technique. Consequently, a solution of water-soluble polymer and reactants was prepared, which was then used to coat a 1.0×20 mm quartz rod. Thus, the polymer acted as a bonding agent and matrix, and also offered protection to the reactants incorporated in the coating.

The lightguide coating combination can be chosen to provide a coating with a refractive index that is either higher or lower than the rod (guide) and which can be either a water or non-water based polymer, depending upon its compatibility with the desired chemical reactants. A lower refractive index is normally used in optical guide applications and would result in the mechanism illustrated (Fig. 1a).

Using the higher refractive index coating, the mechanism shown in Fig. 1b would be operative. Although either approach can be used, the mode shown in Fig. 1a would result in lower sensitivity since the evanescent wave interactions occur only in the region of the rod-coating interface. In either of the mechanisms shown in Fig. 1b, the radiation is transmitted

through the entire coating and so allows solid state spectrophotometric measurements *in situ*.

To measure the light transmittance, an instrument was designed and constructed to provide quantitative analytical measurements. This 'gradient light analytical detector' (GLAD) will accommodate glass rods of 0.9–1.3 mm in diameter, and either 10 or 20 mm long (Fig. 2).

A detailed view of the GLAD optical system is also shown in Fig. 2, with rod dimensions exaggerated to illustrate the basic instrumental operation. The substage condenser converts the collimated beam into a strongly converging hollow cone of light. The hemispherical lens and circular aperture couple the light into the rod. After multiple reflections within the rod, the light emerges at the upper face and is scattered by a diffuser, part of the light going into the silicon photodiode detector. The photodiode is operated in the photovoltaic mode, the operational amplifier acting as a current sink to minimise the voltage across the diode. The amplifier output is a low impedance voltage, proportional to the input current over a range of 10^{-11} – 10^{-3} A.

To demonstrate the concept, we used the technique for the determination of microgram quantities of sodium cyanide in a cyanide-picric acid system. A solution was prepared to contain 1% by weight of polyvinyl alcohol and 0.1% by weight of sodium picric acid, which is known to respond selectively to $(CN)^-$ (ref. 3). This solution was then used to coat the surface of the rods uniformly when the ends were protected.

The light transmission of the dry, coated lightguides was measured before reaction. Known amounts of cyanide ion, in the form of sodium cyanide, were applied to the outer surface of the guides using a 5 μ l Eppendorf pipette, the rods were dried, and the transmission was measured again. The percentage transmission, based on the initial reading before exposure, was then plotted as a function of the $(CN)^-$ concentration (Fig. 3).

The reaction between the cyanide ion and the picric acid changes the refractive index and absorption coefficient of the coating. The resulting change in light transmission through the guide is proportional to the concentration of the cyanide species. The multiple internal reflections enhances the sensitivity, in that a small change in the optical characteristics of the coating

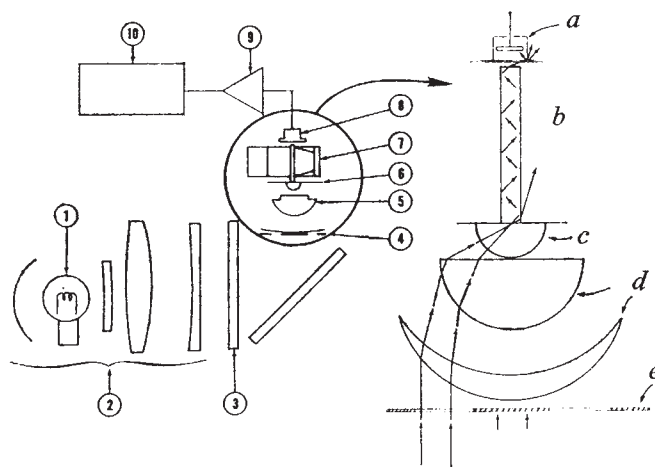


Fig. 2 Block diagram of the gradient light analytical detector (GLAD). 1, A tungsten filament lamp light source; 2, a condenser system to produce nearly collimated light; 3, a filter for wavelength selection; 4, an annular aperture to block axial light rays; 5, a condenser to produce a hollow cone of light rays; 6, coupling hemispheres and apertures to couple large angle rays into the rod; 7, a rod mount to position rods accurately with respect to the aperture while presenting a minimum of surface contact; 8, a silicon photodiode detector; 9, an operational amplifier operating as a 'current-voltage' converter; 10, a three-digit digital voltmeter for relative transmittance readout. a, Detector; b, rod; c, hemisphere; d, condensers; e, annular aperture.

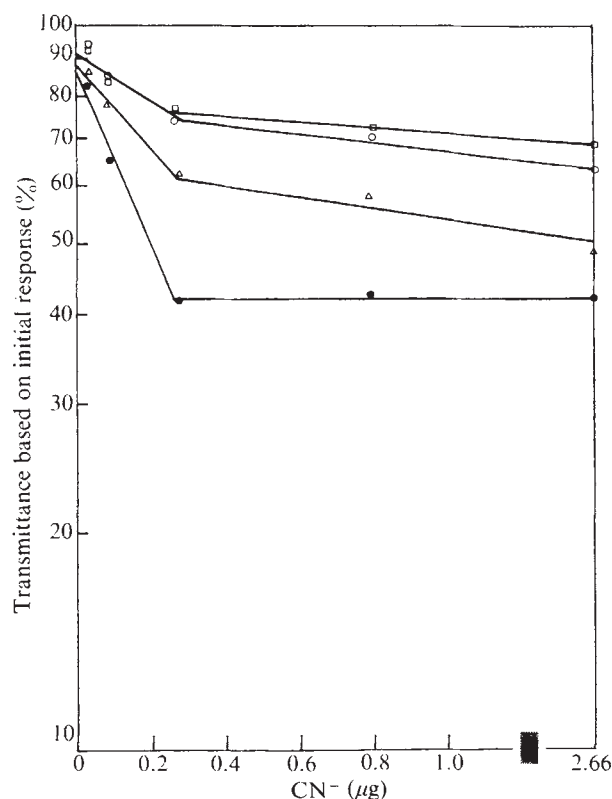


Fig. 3 Concentration of $(CN)^-$ against transmittance. $\square$, Red filter; $\circ$, achromatic light; $\triangle$, orange-yellow filter; $\bullet$, green filter.

causes a large change in the light transmitted through the system. A green filter provides the best sensitivity, as can be anticipated because of the reddish-brown colour of the reaction product; further, Beer's law is obeyed over the expected range of concentration.

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Implications of drought for vertebrate fossil assemblages

THE profound effects of drought on modern ecosystems is widely appreciated. However, little attention has been paid to the palaeontological and geological consequences of drought; the few works¹ which discuss drought in these terms are general. I wish to describe a sequence of events that may occur during a drought and the taphonomic implications of these events.

It is well known that factors such as habitat preference of the animals in question^{2,3}, the degree of disruption of bones by

carnivores⁴, and the environmental setting⁵ influence the likelihood of preservation of thanatocoenoses. My preliminary observations of the Ethiopian drought during 1973 indicate that these factors and many others are affected considerably by drought. I suggest that assemblages of vertebrates that die during a drought may have a disproportionate probability of being represented in the fossil record and that such assemblages can be recognised by the characteristic alterations of an ecosystem by drought.

Tannehill⁶ offers a useful definition of drought as a period of deficient rainfall which is seriously injurious to local vegetation. He avoids defining drought in terms of the amount of rainfall, since what is normal rainfall in one area may be deficient in another. I describe three hypothetical phases of progressive severity of drought. Although the effects of a normal dry season may be the same as some of those described for these phases, it is the complex of effects that indicates an abnormal period of dryness—drought—as opposed to a normal dry season.

In phase I, mild drought, rainfall is less than usual. New vegetation withers quickly or fails to grow; large migratory herbivores move in search of better pastures and more water; seasonal waterholes and streams dry up; mortality is elevated above normal attritional levels, especially among younger individuals.

In phase II, severe drought, rainfall continues to be deficient. Permanent rivers and lakes are shrinking; caliche forms in the alkaline soil, evaporites precipitate and cracks develop in the beds of dried-up rivers and waterholes. Animals respond to the drought according to the frequency with which they need to drink water; thus highly mobile animals with moderate water needs may migrate out of the area. Those animals that need to drink at least once a day congregate along the remaining waterways regardless of their normal habitat preference. All edible vegetation within a critical distance of water is soon eaten, so only those animals that rarely drink water can travel far enough from water to find forage. Many animals die of starvation near major water sources. I observed numerous carcasses within 50 m of the Awash River in Ethiopia; thus the presence of a river or lake near a fossil assemblage in no way indicates the absence of drought. Carnivores may migrate into the area because of the easy availability of food. Observations of hyenas in the Awash suggest that even carnivores which habitually consume bones alter their habits during a drought and eat soft tissues instead; thus skeletons are rarely disturbed by the carnivores during this phase.

In phase III, extreme drought, normal rainfall is still absent. Large lakes and rivers may dry up, leaving huge numbers of dead fish, amphibians and other water-dwelling species. The few herbivores crowd around shrinking pools of water and die struggling to drink. Local extinction of plants and animals is common; extinction may occur on the species level as well. All but the most drought-resistant plants have died. The soil moisture and organic content are low; loose sand may be blown into dunes. If phase III continues long enough, the environment may be permanently altered into desert or semi-desert; in this case, the situation is no longer one of drought. The plants and animals which originally inhabited the area do not return and colonisation by organisms adapted to desert conditions occurs.

For several reasons, a large runoff is likely when rain recurs following phase II or III. The land surface is denuded of vegetation that normally traps and holds water. Caliche impedes the passage of rainwater into the soil, further increasing runoff. Both intermittent and permanent rivers may fill and overflow, transporting and depositing large quantities of sediment. Skeletons lying in or near river or lakebeds are covered rapidly by these sediments; fossilisation may begin.

So far, the few drought assemblages that have been recognised fall into phase III (ref. 7), probably because the effects of this phase are more easily recognisable than the more subtle effects of phase I or II. Phase I assemblages are difficult to distinguish from assemblages resulting from other catastrophes. Phase II

droughts, however, alter the ecosystem sufficiently to be identified. Since failure to recognise a phase II assemblage may seriously distort a palaeoecological reconstruction, it is important that phase II assemblages be distinguished. The following criteria may be used to identify phase II assemblages except those criteria specified as characterising phase III assemblages. No single criterion should be considered as diagnostic in itself, nor will every phase II assemblage exhibit all of these criteria.

The palaeontological criteria are as follows. (1) Since many animals die of starvation rather than thirst, they are buried close to major water sources. (2) The age distribution of individuals killed by drought is indicative of catastrophic mortality^{1,8}. Many young individuals die in phase I; those dying in phase II are likely to be in their prime. (3) Many skeletons are preserved partially or fully articulated because carnivores have not disturbed the bones. Because the palaeo-environment lacks moisture, soft tissues of carcasses are dried and help to keep joints intact⁹. (4) Animals of disparate habitat preference are found in association. Most species are highly dependent on water. (5) In phase III, aquatic or semi-aquatic animals die concentrated in small areas. (6) In phase III, animals such as crocodiles and lungfish die during aestivation. The geological criteria are as follows. (1) Fine-grained strata associated with the fossils show mud cracks. (2) Evaporites and caliche are commonly associated with the fossils but are uncommon in other strata of the deposit. (3) Overlying sediments indicate rapid deposition.

Further study of modern droughts will undoubtedly modify these criteria. Even in their present form, however, they may be useful in re-examining previously described fossil assemblages for which other causes of death have been proposed. On reconsideration, assemblages that include numerous herbivores found in death poses and located near major water sources—such as the *Leptomeryx* assemblages described by Clark and Guensberg¹⁰—may prove to be drought assemblages. Although it has been speculated before⁷ that drought may be related to the extinction of some species, these criteria will enable more certain identification to be made of such extinctions. Finally, if the geological criteria are correct, palaeontologists may be able to find new sites by surveying areas near water sources and characterised by caliche, evaporites and mud cracks.

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Hypsilophodon and *Iguanodon* from the Lower Cretaceous of North America

WE describe here ornithomimid dinosaur remains from the Lower Cretaceous of western North America, which can be referred to the European genera *Hypsilophodon* and *Iguanodon*. The occurrence of these remains indicate that a land connection existed between Europe and North America at the end of the Jurassic.

Though *Hypsilophodon* and *Iguanodon* are well known from Europe and northern Africa (Fig. 1a) no trace of either genus has yet been reported from North America and the only possible Neocomian ornithomimid described from that continent is from the Lakota Sandstone of South Dakota and is referred to *Camptosaurus*¹, a genus well known from the older Morrison Formation (Upper Jurassic). *Camptosaurus*¹ is very different from both *Hypsilophodon*² and *Iguanodon*³. No material of *Iguanodon* is identifiable in the extensive collections from the Morrison Formation of central North America and all the hypsilophodontid material is referable to two genera⁴, *Laosaurus* and *Dryosaurus*. An extensive dinosaur fauna is known from the Cloverly Formation of Montana and Wyoming⁵ but this is younger (Aptian–Albian) than the Wealden, and the iguanodontid *Tenontosaurus*⁶, the only Cloverly ornithomimid described,

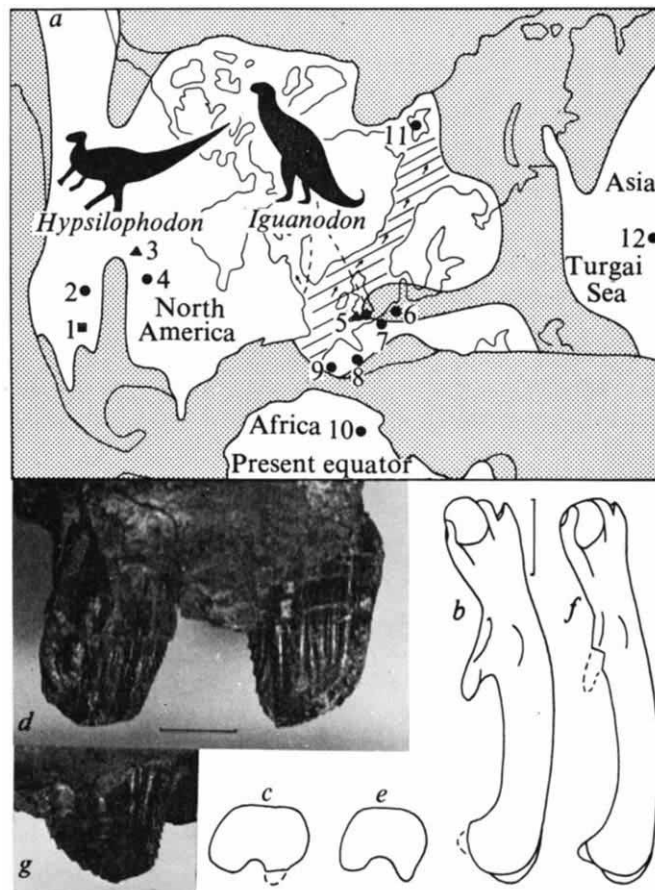


Fig. 1 a, Localities and distribution of land in part of the Northern Hemisphere during the early Cretaceous, after Cox¹⁷, — reconstructed coast lines; —, present coast lines; ---, northern extent of North Atlantic, after Dietz and Holden²²; →→→, circulation currents, after Berggren and Hollister²¹; cross hatching, epicontinental sea, after Hallam²⁰; ▲, *Hypsilophodon* localities; ●, *Iguanodon* localities; ■, *Tenontosaurus* locality; 1, southern Arizona; 2, south-eastern Utah; 3, western South Dakota; 4, eastern Nebraska; 5, southern England; 6, Belgium; 7, France; 8, Spain; 9, Portugal; 10, Tunisia; 11, Spitzbergen; 12, eastern Gobi Desert (shifted slightly left). b and c, left femur ($\times 0.2$) of *Hypsilophodon* sp. (American Museum of Natural History No. 2585), collected in Lakota Sandstone about 38 m above the Morrison Formation 4.8 km north of Piedmont, South Dakota; b, medial view, c, distal end; d, lateral view of posterior part of right maxilla ($\times 1.0$) of *Iguanodon* sp. (Brigham Young University Earth Sciences Museum No. 2000), collected 1.2 km east of Dalton Well, Grand County, Utah; e and f, left femur ($\times 0.5$) of *Hypsilophodon foxii*, Wealden of Isle of Wight; e, distal end; f, medial view; g, lateral view of posterior tooth of left maxilla⁹ ($\times 1.0$) of *Iguanodon mantelli* (British Museum (Natural History) No. R754), from Wealden (Tilgate Forest) of Cuckfield, Sussex. Scale lines represent 5 cm (b, c, e, and f) or 1 cm (d and g); although the North American *Hypsilophodon* femur is much larger than the European specimen figured here, it falls within the size range of the genus¹.

is only distantly related to *Iguanodon*. Consequently, the specimens described here (Fig. 1b, c and d) represent the first material of the characteristically European genera *Hypsilophodon* and *Iguanodon* to be recognised from North America.

The left femur (Fig. 1b and c), from the Lakota Sandstone of South Dakota, is very similar to the femora of *Hypsilophodon foxii*² (Fig. 1e and f). The other hypsilophodontid femora from North America differ from these in several respects. In *Hypsilophodon* itself there is a shallow cleft separating the proximal trochanters (Fig. 1b and f) whereas in *Laosaurus* (= *Nanosaurus rex*)⁶ and *Dryosaurus* the cleft is deep; in *Parksosaurus* (Upper Cretaceous), on the other hand, the cleft is apparently absent⁷. The distal end of the femur of *Hypsilophodon* (Fig. 1c and e) has practically no anterior intercondylar groove; this groove is, however, well developed in *Dryosaurus*, where the posterolateral condyle is thin, as it is in *Parksosaurus*⁷.

The posterior part of the right maxilla (Fig. 1d) was collected from the Lower Cretaceous Cedar Mountain Formation⁸ of south-eastern Utah. The teeth are very similar to some of those from *Iguanodon*⁹ (Fig. 1g) from Britain, and the Utah specimen is, therefore, probably referable to *Iguanodon* (D. Norman, personal communication). The thickly enamelled lateral surface of the crown has a prominent keel (Fig. 1d), whereas in *Tenontosaurus* only dentary teeth bear such a keel⁵. The presence of a deep vertical depression on the anterior and posterior edges of the root and base of the crown also serves to distinguish these teeth from those of *Tenontosaurus*⁵ and *Camptosaurus*¹. The distal end of a right femur from the same area as the maxilla has a very deep anterior intercondylar groove, as in *Iguanodon*³; again, this is in marked contrast to the shallow groove present on femora of *Tenontosaurus*⁵ and *Camptosaurus*¹. The distal end of a femur from the Dakota Sandstone of Nebraska¹⁰ can also be referred to *Iguanodon* on account of a very deep anterior intercondylar groove. The distal ends of the femora of certain hadrosaurs¹¹ are also similar to those of *Iguanodon*³, but hadrosaurs are not known to occur so early, the oldest known specimen from North America being late Cretaceous in age (Santonian, Mississippi)¹², though older records come from the Cenomanian of England¹³ and the Aptian-Albian of Mongolia¹⁴.

The North Atlantic started to open during the early Jurassic¹⁵ with a second stage of expansion, corresponding to the Bermuda discontinuity, probably ending sometime during the late Jurassic or early Cretaceous¹⁵. The presence of a 'transatlantic' land connection during the Middle Jurassic is clearly shown by the great similarity of the Upper Jurassic dinosaur faunas best known from the Morrison Formation of North America and the Tendaguru of Tanzania¹⁶⁻¹⁸. There are, however, no dinosaurian genera common to the Wealden Beds of Europe and either the Arundel Formation¹⁹ (Neocomian) of Maryland or the Cloverly Formation⁵. The specimens of *Hypsilophodon* and *Iguanodon* described here show that the presumed absence of these genera from North America reflect the lack of material from beds of appropriate age and/or ecology.

Reconstructions of the northern limit of the North Atlantic at about the time of the Jurassic-Cretaceous transition vary (Fig. 1a) but, taking into account epicontinental seas, the North Atlantic probably connected with the Arctic Ocean at that time^{20,21}. Epicontinental seas are, however, shallow and liable to retreat and advance locally, so they are not such an absolute barrier to terrestrial animals as are deep oceans. The presence of two characteristically European genera in North America indicates a northern land route between these two areas at about, or immediately before the Jurassic-Cretaceous transition. Future discoveries in the Neocomian of North America will probably increase the number of genera common to both areas. During the early Cretaceous the Mid-Continental Seaway of central

North America (Fig. 1a) had not extended far enough north to separate two distinct dinosaurian faunas in the northern hemisphere (Asiaamerica, Euramerica), as occurred in the Upper Cretaceous¹⁷.

The specimens discussed here will be fully described elsewhere²³.

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Membrane augmentation in freezing tolerance of plant cells

THE question of the special physiology and biochemistry of growth and adaptation of plants to low temperature has been raised¹ with reference to the significance of the increased unsaturation of fatty acids observed in such plants. Interest in the phenomenon of increased unsaturation with exposure to low temperatures, first documented by Hilditch², has received impetus from reports^{3,4} that membranes and membrane lipids of plants sensitive to chilling change from a liquid-crystalline to a gel structure when exposed to low temperatures, whereas those from plants resistant to chilling undergo no such phase change. The implication was that the less unsaturated membrane lipids of cells or organelles from plants which are sensitive to chilling were less fluid at lower temperatures and consequently lost normal function at such temperatures. Reports of increased fatty acid unsaturation in phospholipids of plant tissues^{5,6} which are tolerant of chilling lend credence to these views. The apparently reasonable extrapolation from these findings that has been made⁷⁻¹¹, and that was inferred by Moore¹, is that the resistance of plants to injury from actual freezing may also be derived from changes in unsaturation and fluidity of membrane lipids. This theory warrants closer examination. The two stresses of chilling and freezing are quite different; chilling resistance is an adaptation to low temperatures above freezing and principally involves the interaction between metabolic function and structure, whereas freezing resistance is tolerance to the dehydrative stresses of crystallisation of ice, and seems to be largely structural in nature^{12,13}. It is reasonable to expect, therefore, that the underlying chemistry of resistance to chilling and freezing would be different. Our findings indicate that this is the case.

Two conclusions can be drawn from our results (Table 1). First, in the transition of the living bark cells from the summer unhardy (LD₅₀ -10 °C) to the winter hardy (LD₅₀ > -196 °C)

Table 1 Fatty acid composition and PC:PE ratios of summer and winter living bark tissues of the black locust tree

Tissue	Phospholipid	Fatty acid composition (mol %)					Δ/mol^*	PC:PE†
		16:0	18:0	18:1	18:2	18:3		
Summer (LD ₅₀ -10 °C)	Total	21.4	1.7	5.8	62.8	8.3	1.6	1.5
	PE	23.7	1.5	4.8	64.1	5.9	1.5	
	PC	15.7	2.2	6.7	63.1	12.2	1.7	
Winter (LD ₅₀ > -196 °C)	Total	19.8	0.4	2.3	69.7	7.7	1.6	1.6
	PE	23.2	0.4	1.8	69.1	5.5	1.6	
	PC	12.8	0.5	2.2	74.5	9.9	1.8	

Phospholipids extraction, separation and fatty acid determination were performed according to methods described previously¹¹.

* $\Delta/\text{mol} = 1.0 \times (\% \text{ monoene})/100 + 2.0 \times (\% \text{ diene})/100 + 3.0 \times (\% \text{ triene})/100$.

†By mol.

condition no significant change occurs in the fatty acid unsaturation of either of the two major phospholipids, phosphatidylcholine and phosphatidylethanolamine. These results extend our finding¹⁴ of a lack of change in unsaturation in both the total lipids and combined phospholipid fractions. Second, no significant shift occurs in the proportions of the two major phospholipid classes. These results agree with those of Yoshida and Sakai¹⁵ for the poplar tree. Changes in fluidity of membranes arising merely from increased unsaturation or from a shift in phospholipid classes cannot, therefore, be the factors responsible for the development of extreme freezing tolerance. In contrast to trees, herbaceous plants do undergo large increases in unsaturation during hardening^{9,10}, which must be a result of exposure to the low temperature regimes required for hardening since winter wheat cultivars of contrasting hardness exhibit the same degree of unsaturation¹⁶. It is also interesting that the relative unsaturation of tree cells in their hardest condition (LD₅₀ > -196 °C) is still less than that of cereals in their most tender state (LD₅₀ -2 °C).

So far the only change we have observed in the locust bark cells that is consistent with the development of extreme freezing tolerance is the augmentation of membranes. Table 2 demonstrates the quantitative augmentation of membrane components on a DNA or unit cell basis. The development of

Table 2 Augmentation of phospholipid and protein in insoluble homogenates of summer and winter black locust bark tissues.

Tissue	Temperature at		Phospholipid/DNA	Protein/DNA
	LD ₅₀			
Summer	-10 °C		4.7	14.0
Winter	> -196 °C		11.1	33.0

Insoluble homogenates were prepared as described in ref. 11. DNA was measured by estimation of adenine content after Schmidt-Thannhauser extraction and removal of RNA (J. S. and D. S., unpublished). LD₅₀ refers to the temperature at which 50% of the cells were killed. Values presented represented means of three replicates.

tolerance to freezing must, therefore, be associated directly with this increase in membranous material as we have also been able to produce, by starvation of these cells graded levels of resistance to freezing which correlate directly with graded concentrations of phospholipid¹³.

We suggest, therefore, that at least one of the mechanisms of resistance to freezing, and probably the major one in these trees, involves the augmentation of membranous material in their cells. The biochemical and biophysical mechanism by which membrane augmentation enables the cell to accommodate to the stresses of extreme freezing are being investigated further.

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Superoxide dismutase in photosynthetic organisms provides an evolutionary hypothesis

Two years ago it seemed that a unifying hypothesis accounting for the distribution of superoxide dismutase (SOD) in different organisms had been achieved. Thus the enzyme was present only in aerobes and aerotolerant anaerobes, but absent from obligate anaerobes¹; eukaryotes contained primarily the cuprein (Cu-Zn) type of dismutase (cyanide-sensitive) whereas in prokaryotes the enzyme had either manganese or iron at the catalytic site (cyanide-insensitive); finally, the presence of a manganodismutase in the matrix space of mitochondria was interpreted² in terms of a polyphyletic origin for eukaryotes with independent evolution of SOD in the prokaryotes (Mn-SOD) and proto-eukaryotes (Cu-Zn SOD) before the postulated symbiotic event.

Several subsequent findings, however, have justified the cautionary nature of Fridovich's closing remarks to his hypothesis³. Lavelle *et al.*⁴ found only a single, manganese-containing SOD in the luminous fungus *Pleurotus olearius*; Lindmark and Müller⁵ reported cyanide-insensitive SOD activity in two aerotolerant protozoa; Puget and Michelson⁶ isolated a bacteriocuprein from *Photobacterium leiognathii*; Hewitt and Morris⁷ detected SOD in a wide range of obligate anaerobes; and, finally, Asada *et al.*⁸ noted the absence of cyanide-sensitivity in SOD from all algal phyla studied.

We have made an electrophoretic survey of the occurrence of SOD in photosynthetic organisms, both prokaryotic (bacteria and blue-green algae) and eukaryotic (red and green algae) (Fig. 1), and have confirmed the individual results of Hewitt and Morris and of Asada *et al.* All the organisms we studied contain cyanide-insensitive SOD activity and, in the case of the green alga *Codium*, a part of this activity has been localised in the chloroplast stroma. This contrasts with the situation in higher plants in which, so far, only the Cu-Zn enzyme has been found⁹⁻¹¹. We wish to suggest a new scheme for the evolution of photosynthetic organisms, relating the findings on SOD to the con-

ditions in which the initial symbiotic events are thought to have taken place.

The bacteriostatic effect of oxygen on *Chlorobium* and *Chromatium* may result because SOD cannot prevent the inactivation of certain enzymes vital to their metabolism (for example, Fe-S proteins). We must presume either (1) that SOD evolved independently in obligate anaerobes or (2) that SOD evolved before oxygenic photosynthesis as a result of the Urey effect, that is the production of oxygen by ultraviolet photolysis of water¹². There is no general agreement on the level of atmospheric oxygen which this would have produced¹³, but, if the enzymes from *Chlorobium* and *Chromatium* are as similar as the gels suggest, SOD must have antedated the divergence of these lines. It might be argued that some of the multiple bands of activity (especially those of *Chromatium*) do not represent genuine isozymes and are artefacts of electrofocusing or staining; however, the three activities of aerobically-grown *Rps. spheroides* extracts can be separated by ion exchange chromatography and give consistent single bands on gels.

The following scheme for the evolution of eukaryotic algae is constructed from the premise of the symbiotic origin of subcellular organelles, although the experimental findings are not incompatible with alternative hypotheses¹⁴. The sequence of events would have been initiated by the acquisition, by a photosynthetic bacterium, of a water-splitting enzyme system linked to the photosynthetic electron transport chain, resulting in an oxygenic photosynthesis similar to that of present-day algae and plants. We have proposed that this enzyme, which contains manganese, may originally have had a dual role, functioning also, in a soluble form, as a SOD so that oxygen-evolving and oxygen radical detoxification mechanisms could have appeared simultaneously¹⁵. An immediate effect of this photosynthetic oxygen evolution would have been to force an aerobic respiratory metabolism on primitive nitrate-reducing bacteria¹⁶. Certain lines of these primitive aerobes may have evolved

mechanisms for phagocytosis and entered into a symbiotic relationship with primitive blue-green algae, resulting in a cyanome-like organism^{17,18}. This would have been to the benefit of both partners, the blue-green alga would have an environment of lower oxygen tension and the host cell would have a stable source of oxygen independent of the algal colonies.

A second symbiotic association, between a cyanome and an aerobic prokaryote would, when stabilised, produce an organism similar to a present day red alga. de Duve¹⁹ and Hall²⁰ have discussed how it may have benefited an aerobic host cell to enter such a relationship with another aerobe. Indeed, Hall makes the point that, if the host cell already contained enzymes duplicating many of the functions of the endosymbiont, then the latter may have become dependent on the host enzymes, thus explaining the nuclear encoding of mitochondrial proteins such as cytochrome *c* and SOD²¹.

Two important points must be made about the environment in which these events took place. Hall¹⁶ considers that a photosynthetic population large enough to produce a net increase in atmospheric oxygen could not have been achieved without the evolution of the environmentally less demanding eukaryotic algae. Thus the evolution of quite advanced forms of eukaryotes can be envisaged as taking place within a restricted, oxygen-rich environment, although the atmosphere at large remained anaerobic. Weyl²² has proposed that the tropical thermocline could have fulfilled this role, vertical diffusion of oxygen between the density layers of the ocean being greatly restricted. Second, it has been pointed out that, in a reducing atmosphere, copper would have been largely trapped as insoluble cuprous sulphides²³. Following the scheme outlined above, soluble copper would not have become available until after the evolution of eukaryotic algae, so that the earliest eukaryotes would not have contained Cu-Zn SOD.

The acquisition of Cu-Zn SOD by certain lines of eukaryotic organisms must now be considered. Given that closely related organisms survive whether or not they contain Cu-Zn SOD (for example, higher plants and algae, *Neurospora* and *Pleurotus*, *Photobacterium leiognathii* and other *Photobacterium* spp.), it can be inferred that there are no important functional differences between the types of SOD. We suggest that, while the potentialities of copper as a catalyst in biological systems were being explored (for example, in cytochrome oxidase and plastocyanin) a copper protein evolved in one or two lines which was, fortuitously, an efficient SOD and that this enzyme coexisted with, or in some cases replaced, the existing dismutase. Such a possibility is perhaps not unlikely in view of the SOD activity exhibited by quite simple copper-peptide chelates²⁴ and by galactose oxidase²⁵. The reactions between cuprein copper and various catechols²⁶ may be an indication of the now relict function for which the cupreins were originally evolved. Bacteriocuprein can be regarded as such a phenomenon occurring independently in a prokaryote—the only case so far detected because prokaryotes in general make less use of copper in enzymes²⁷.

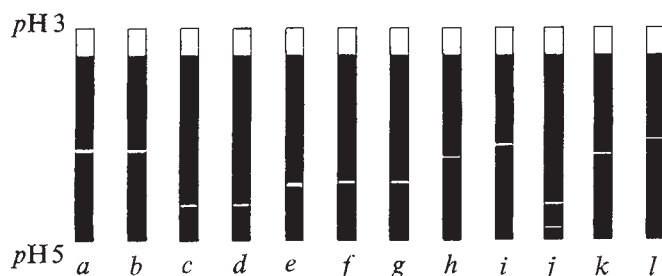
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Fig. 1 Polyacrylamide gel isoelectric focusing of SOD from photosynthetic organisms. SOD in soluble extracts of the organisms was partially purified by fractionation with ammonium sulphate (50–90% saturation) followed by dialysis. These crude extracts were assayed with or without 1mM KCN by the photochemical method of Beauchamp and Fridovich²⁸ (because of the instability of cyanide this was prepared on the day of use); then 2–10 U of SOD were applied per gel which was run²⁹ and stained²⁸ as described. See also ref. 11. Ampholytes were obtained from LKB Ltd. From left to right, the organisms are: a, *Chlorobium thiosulphatophilum* (green bacterium); b, *Chromatium D* (purple, sulphur bacterium); c, *Rhodospseudomonas spheroides* (purple, non-sulphur bacterium) anaerobically grown; d, *Rps. spheroides*, aerobically grown; e, *Nostoc muscorum* (blue-green alga); f, *Anabaena cylindrica* (blue-green alga); g, *A. flos-aquae* (blue-green alga); h, *Spirulina platensis* (blue-green alga); i, *Porphyridium cruentum* (red alga); j, *Scenedesmus obliquus* (green alga); k, *Codium fragile* (coenocytic green alga) chloroplast stromal extract; l, *C. fragile*, cytoplasmic extract. In the light of present knowledge, it has been assumed that insensitivity to inhibition by cyanide is a valid criterion of the absence of the cuprein-type of SOD from crude extracts. Nevertheless, until several of these enzymes have been characterised, the possibility of the existence of a class of cyanide-insensitive, copper-containing dismutases should be borne in mind.



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Cross-modal matching in the monkey after discrete temporal lobe lesions

ETTLINGER¹, reviewing the literature on cross-modal matching in primates, concluded that whereas man and chimpanzees seemed to be capable of such matching, the evidence concerning the monkey was ambiguous. Cowey and Weiskrantz², however, reported a method which resulted in a successful demonstration of a tactile-visual cross-modal match in normal rhesus monkeys. Their success seemed to result from the use of stimulus shapes made from powdered monkey diet, which the animal could eat. Such a training paradigm would be more natural—and therefore presumably easier—for the animal to master. In their experiment, monkeys were allowed to sample ten palatable and ten different unpalatable shapes (treated with quinine) in the dark; subsequently, the monkeys were given a choice between one palatable and one unpalatable shape, presented with the house light switched on. The monkeys chose the palatable shape significantly more often than the unpalatable, showing that a cross-modal match had occurred.

We have studied the effects of discrete temporal lobe lesions on cross-modal matching ability, using the Cowey and Weiskrantz method. Our subjects included three monkeys (two *Macaca mulatta* and one *Papio papio*) with foveal prestriate (FPS) lesions, two (one *M. mulatta* and one *P. papio*) with posterior inferotemporal (PIT) lesions and two monkeys (*M.*

mulatta) with anterior inferotemporal (AIT) lesions. These lesions are shown in Fig. 1. Each animal had an extensive history of testing on a visual delayed matching to sample task using colours, ellipses and curves as stimuli. These experiments showed that the FPS lesion produced no impairment, the PIT lesion resulted in animals with perceptual deficits and the AIT lesion produced an impairment that was significantly influenced by delay. In addition to these animals, two monkeys (*M. mulatta*) served as unoperated controls.

We used a standard Wisconsin general test apparatus (WGTA) and the transport cage which housed the monkeys was constructed so that shapes could not inadvertently become lodged within sight of the animal. Stimuli were prepared as follows: the positive shapes were made from Dixons powdered monkey diet (41-B) made into a dough like consistency with water (a small quantity of sugar was added to render them even more palatable), and then cut into various shapes with pastry cutters, and dried in a warm oven. Negative (unpalatable) shapes were made in a similar manner, except that no sugar was used, and a 5% aqueous solution of quinine dihydrochloride was used instead of water. The eighteen shapes made are illustrated in Fig. 2.

The monkeys were first trained to accept the experimental food in the dark in the WGTA. They were then presented with ten palatable and ten unpalatable shapes, in a counterbalanced design. For example, one monkey from a group received ten palatable crosses and ten unpalatable disks, while the other monkey received ten palatable disks and ten unpalatable crosses. All twenty shapes were presented simultaneously on a tray; the monkey was allowed to sample them at his leisure, typically taking about 10 min to do so. About 90% of the unpalatable shapes were discarded intact. The bottom of the transport cage was then covered with a cardboard sheet to prevent the animal from subsequently noticing any discarded stimuli, and the house light was switched on. For the next 20 s, the animal was shown one palatable and one unpalatable shape. Both these stimuli were manipulated by the experimenter so that the animal saw them from all possible angles. They were then placed side by side on a tray, and presented to the animal, who was permitted only one choice. This procedure was repeated daily, with a different pair of shapes being used on different days. These shapes were recombined after all nine pairs had been presented. In all, twenty choices per animal were recorded.

At the conclusion of the cross-modal study, all animals were given visual discrimination problems, using the palatable and

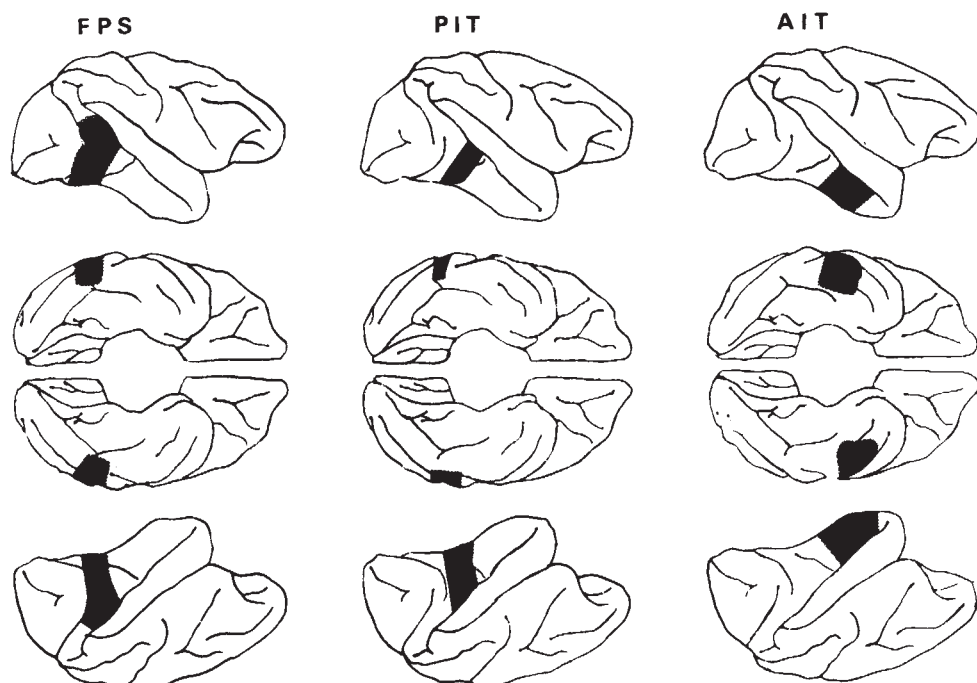


Fig. 1 Common (black) and maximum (stippled) extent of the lesions. For FPS $N = 3$, for PIT $N = 2$ and for AIT $N = 2$. The FPS lesion was designed to extend from the lower limits of the lunate sulcus to the superior temporal sulcus and from the lunate sulcus ventrally to the inferior occipital sulcus, thus including part of the foveal representation of Zeki's³ area V4. The AIT lesion extended anteriorly from the inferior occipital sulcus, with a lateral extent from the superior temporal sulcus to the ventral temporal lobe. The AIT lesion was placed in the anterior ventral temporal lobe cortex. The PIT plus AIT damage together constitute the traditional inferotemporal or TE lesion.

Table 1 Performance of individual subjects expressed as percentage correct on cross-modal matching and visual discrimination tasks

Animal and lesion	Cross-modal percentage corrects	<i>P</i>	
		binomial two-tail test	Visual discrimination percentage corrects
Friend FPS	60		45
Eunuch FPS	35	0.8	70
Judge FPS	50		68
Schizophrenic PIT	45	0.65	68
Man PIT	60		78
Fool AIT	75	0.0046	73
Cockatoo AIT	75		73
Jumper N	75	0.0114	73
James N	70		76

unpalatable shapes. Each animal had ten trials daily, using different pairs of stimulus shapes on different days, for a total of 4 d. This experiment was undertaken to see if the impairment in cross-modal matching could be attributed to a gross perceptual and/or mnemonic dysfunction within the visual modality.

Table 1 shows the results on cross-modal matching. The ability of normal monkeys to make a cross-modal match correctly, as determined by Cowey and Weiskrantz, was confirmed ($P = 0.0114$, binomial two-tailed). Both FPS and the PIT lesions impaired cross-modal matching ($P = 0.8$ and 0.65 respectively), whereas the AIT lesions did not ($P = 0.0046$).

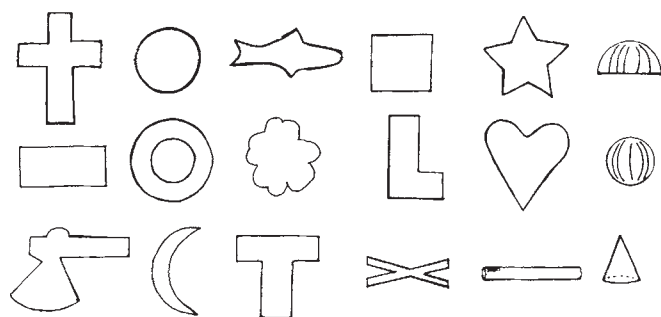


Fig. 2 Outlines of the eighteen shapes used, as viewed vertically. Their size can be judged from the first shape (cross), which was 5 cm along its short axis.

Statistical comparison indicated that the groups differed (information statistic⁴, $2\hat{I} = 11.728$, d.f. = 3, $P < 0.01$) and that this difference was between the AIT and the normal against the FPS and PIT groups ($2\hat{I} = 11.558$, d.f. = 1, $P < 0.001$). Furthermore, the differences between the AIT and the normals and between the FPS and PIT were not significant ($2\hat{I} = 0.170$, d.f. = 2, $P > 0.9$). On the right of Table 1 the overall percentages correct on the 40 trials on four discrimination tasks are presented, along with group significance levels, showing that none of the groups was impaired. A further analysis of the results on the visual discrimination tasks is presented in Table 2. An analysis by lesion group of the number of correct and incorrect choices on trials 2–6 on the four problems revealed no significant differences in visual discrimination performance (information statistic⁴, $2\hat{I} = 8.88$, d.f. = 15, $P > 0.8$).

The finding that the AIT group succeeded in matching whereas the PIT and the FPS animals failed was somewhat surprising, especially in view of the findings in the delayed matching to sample tasks described above. One plausible explanation stems from the findings of Iwai and Mishkin⁵, that AIT lesions result in a visual mnemonic deficit, although the more posterior lesions such as the PIT and FPS result in perceptual impairments. Furthermore, Iversen⁶ has shown that the visual memory deficit in inferotemporal lesioned monkeys is greatest when visual interference occurs. She found that inferotemporal monkeys kept in darkness after learning a visual discrimination problem were as good as normal monkeys in their ability to remember the task, whereas after new visual learning, retention was

greatly reduced. On this analysis, an AIT lesion would not impair cross-modal matching since much of the task is undertaken in the dark; and interference when the house light is switched on would be minimal, since the animal's attention is fixed on the relevant stimuli. In contrast, a PIT and a FPS lesion may impair the animal by causing a subtle visual perceptual breakdown. We do not think, however, that this is very likely. All our animals were equally good (with the exception of one FPS animal) at solving the visual discrimination problems, ruling out gross visual impairment. Since it is also known that temporal cortical lesions do not cause a tactile impairment (see ref. 7 for a review), the safest conclusion is that PIT and FPS lesions, in contrast to AIT lesions, result in a disruption of the mechanism that mediates a tactile-visual match. Further work is in progress to elucidate the common damage in the FPS and PIT lesions which underlies the reported impairment. It is worth noting that both lesions interrupt visual input to a multi-modal convergence area in the depths of the superior temporal sulcus^{8,9} and also includes partial damage to the sulcus itself. One of us (M.P.) is investigating the effects of bilateral lesions in the superior temporal sulcus, using cross modal matching and related tasks. Preliminary results suggest that there is a tactile visual cross-modal matching deficit.

Table 2 Performance matrix for the visual discrimination tasks

FPS (N = 3)	PIT (N = 2)	AIT (N = 2)	Control (N = 2)	
3 5 5 3 5	3 1 4 4 2	2 3 5 1 2	3 2 2 2 2	Incorrect
9 7 7 9 7	5 7 4 4 6	6 5 3 7 6	3 4 4 4 4	Correct
2 3 4 5 6	2 3 4 5 6	2 3 4 5 6	2 3 4 5 6	Trial No.

This analysis was performed only on trials 2–6, since the animal would of necessity perform at chance on the first trial, and after the sixth trial most problems had been learned. Thus, analysis based on trials 2–6 represents a conservative estimate of performance. The normal controls were tested on only three of the four visual problems.

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Spatial and temporal contrast sensitivity of striate cortical neurones

THE human visual system detects discontinuities in the spatial or temporal pattern of light falling on the retina; it is relatively poor at detecting steady or slowly changing patterns in either domain. These insensitivities to low rates of change in space and time are not independent: a gentle gradient of illumination can be made more visible by moving it, or modulating its brightness in time. Patterns of alternating light and dark bars with a sinusoidal luminance profile across the bars—sinusoidal gratings—are commonly used to study spatial interactions in the visual system. Some of the earliest workers to use these stimuli noticed that human sensitivity to steady sinusoidal gratings of low spatial frequency is poor, but is markedly enhanced if the grating is moved or modulated in time^{1,2}.

It has often been proposed that the lateral inhibitory mechanisms responsible for the system's relative insensitivity to low spatial frequencies operate with a slower time course than excitatory mechanisms, relatively reducing the effectiveness of lateral inhibition during the presentation of time-varying stimuli^{2–6}. Adaptation studies, however, suggest that temporal modulation does not change the spatial frequency tuning of individual spatial filters ('channels') in the human visual system^{7,8}; the increased detectability of temporally modulated low spatial frequency gratings could reflect the activity of a separate population of channels, sensitive to low spatial frequencies and relatively insensitive to steady stimuli^{8,9}.

The available neurophysiological evidence is equivocal. Lateral inhibition in the receptive fields of cat retinal ganglion cells is somewhat slower than excitation from their receptive field centres^{10,11}. But at the next stage of visual processing, the lateral geniculate nucleus, there is no difference between the time courses of excitatory and inhibitory mechanisms¹². We have examined the spatial frequency tuning of single neurones in cat striate cortex to establish whether their tuning is affected by the frequency of temporal modulation: does an increase in the temporal frequency of stimulation enhance a unit's sensitivity to low spatial frequencies?

Adult cats were prepared for electrophysiological recording using techniques described elsewhere¹³. Surgery was performed under short-acting barbiturate anaesthesia (Brietal); for recording, animals were artificially ventilated with a mixture of $N_2O-O_2-CO_2$ (78:20:2). The eyes were stabilised with an intravenous infusion of gallamine triethiodide (Flaxedil: 10–30 mg kg⁻¹ h⁻¹), coupled with bilateral cervical sympathectomy. Pupils were dilated with homatropine, and the corneas were protected with clear contact lenses. Artificial pupils (3 mm diameter) were placed directly in front of the eyes, and supplementary lenses were chosen to focus them on a screen 114 cm distant. The activity of single neurones was recorded with tungsten-in-glass micro-electrodes¹⁴ hydraulically advanced into the cortex through a sealed chamber over a small craniotomy and durotomy. Amplification and display of action potentials were conventional; a standard pulse triggered by each spike was fed to an audiometer, from which the experimenter judged the activity of the cell.

Receptive fields were initially mapped on a tangent screen

using flashed and moving stimuli, and classified according to the criteria of Hubel and Wiesel¹⁵. The dominant eye was chosen, and a cathode-ray oscilloscope, whose mean luminance was 150 cd m⁻², and which subtended 10° × 12.5° was centred on its receptive field; the other eye was covered.

Drifting sinusoidal gratings of different spatial and temporal frequencies, optimised for orientation and direction of movement, were generated on the oscilloscope face by a PDP-11/20 digital computer. The temporal frequency of a drifting grating is the number of cycles of the grating which pass any point on the screen in 1 s; its contrast is the difference between the luminances of its brightest and dimmest points divided by their sum. For any one cell, as many as forty different gratings were used—eight spatial frequencies, each drifting at five different temporal frequencies. The computer presented the gratings once each in random order, and the experimenter adjusted the contrast of each and turned it on and off under computer control until he judged that its introduction and withdrawal caused a liminal change in the activity of the cell. When a threshold had been estimated for each of the stimuli, their order was re-randomised and they were presented again. Four estimates of each threshold were obtained in this way; the standard error of their mean was normally between 0.1 and 0.2 log units.

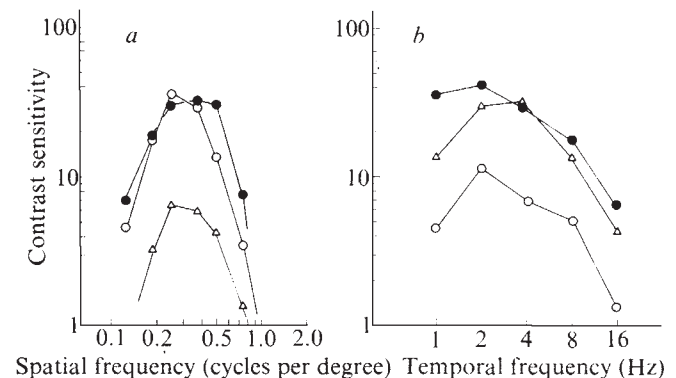


Fig. 1 Spatial and temporal contrast sensitivity functions of a simple cell. *a*, Spatial frequency tuning. The cell's contrast sensitivity as a function of the spatial frequency is shown for gratings drifting at three temporal frequencies. $\circ$, 1 Hz; $\bullet$, 4 Hz; $\triangle$, 16 Hz. *b*, Temporal frequency tuning. The contrast sensitivity as a function of temporal frequency is shown for gratings of three spatial frequencies. Contrast sensitivity is the reciprocal of the contrast at threshold, $(L_{max} + L_{min}) / (L_{max} - L_{min})$. $\circ$, 0.13 cycles per degree; $\bullet$, 0.25 cycles per degree; $\triangle$, 0.5 cycles per degree.

We examined the spatial frequency tuning of 48 units from 10 cats. Of these, 27 were simple, 18 complex and two non-oriented. We examined spatial frequency tuning as a function of temporal frequency in the manner described above in 26 of these units, 15 simple and 11 complex.

Figures 1 and 2 illustrate the results of two such experiments, performed on a simple cell and a complex cell recorded within 70 μ m of each other. Figures 1*a* and 2*a* show that both cells were well-tuned for spatial frequency, and that the general form of their tuning curves was little affected by a 16-fold increase in temporal frequency. Neither the optimum spatial frequencies nor the relative low-frequency sensitivity was systematically changed. Although their overall sensitivity varied considerably with temporal frequency (Figs 1*b* and 2*b*) it is clear that the shapes of their temporal frequency tuning curves at different spatial frequencies were rather similar, apart from a slight tendency for the sensitivity to slow drift rates to be rather less at non-optimal spatial frequencies. This may reflect the fact that the responses of cortical cells to stimuli which are not optimal for the cell are more transient than those to optimal stimuli¹⁶.

The cells illustrated are typical: in no case did we observe

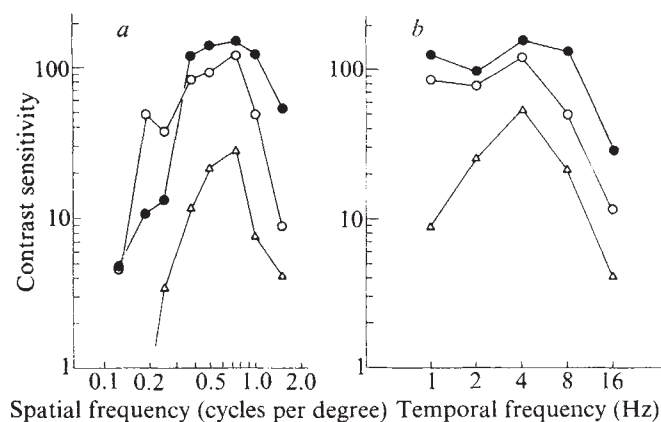


Fig. 2 Spatial and temporal contrast sensitivity functions of a complex cell. *a*, Spatial frequency tuning. $\circ$, 1 Hz; $\bullet$, 4 Hz; $\triangle$, 16 Hz. *b*, Temporal frequency tuning. Conventions as in Fig. 1. $\circ$, 0.38 cycles per degree; $\bullet$, 0.75 cycles per degree; $\triangle$, 1.5 cycles per degree.

any tendency for cells to become less well-tuned for spatial frequency at high temporal frequencies.

The results shown in Figs 1*b* and 2*b* also show that the sensitivity of the units to the velocity of stimulus movement depends on its spatial frequency. Expressed in terms of velocity in degrees per second (temporal frequency divided by spatial frequency), the cells' optima vary by a factor of eight to twelve over the range of spatial frequencies used. This contrasts with the situation when single moving bars are used as stimuli: in that case, the preferred velocity is independent of bar width¹¹.

Our results may indicate that the effects of temporal modulation on human sensitivity to gratings of low spatial frequency cannot be explained by a change in the spatial frequency tuning characteristics of single neurones, unless neurones in the visual cortex of man are different from those in cat. Although we have not observed much variation among the temporal characteristics of striate neurones, it has been reported elsewhere¹⁸ that visual cortical neurones can be subdivided into a sustained group sensitive to high spatial frequencies and a transient group more sensitive to lower frequencies, which may represent the two populations of neurones required to explain the increase in low-frequency sensitivity produced by temporal modulation seen psychophysically.

It is also interesting that, since the sensitivity of units to the temporal frequency of drift does not depend on the spatial frequency of the stimulus, their selectivity in the domain of stimulus movement seems not to be for velocity but for temporal frequency, or the local rate of change in luminance with time.

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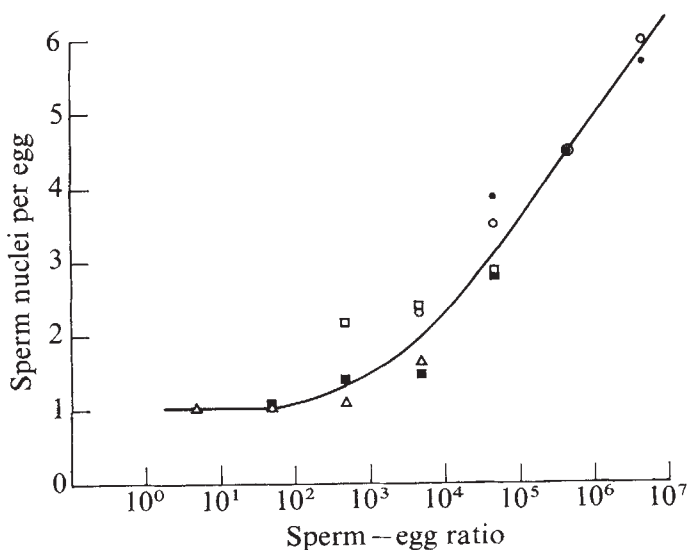
Absence of fast block to polyspermy in eggs of sea urchin *Strongylocentrotus purpuratus*

SEA urchin eggs remain monospermic during fertilisation by excluding the entry of all but one sperm. Two mechanisms have been proposed to account for this exclusion. The first is a rapid alteration of the egg surface, lowering its receptivity to sperm, which occurs within a few seconds of successful contact of the fertilising sperm; this is the so-called fast block¹. This block prevents or reduces further sperm entry for the next 30 s, until the time of the cortical reaction, which establishes the second and permanent block to polyspermy. This permanent or cortical block results from the release of cortical granule proteases, elevation of the fertilisation membrane, and subsequent detachment of sperm. The existence of a cortical block has been well established by a number of investigators²⁻⁴. Although the concept is intuitively compelling, the existence of a fast block has been questioned^{5,6}, and it is supported by only one series of experiments¹.

An opportunity arose to test the hypothesis of a fast block in sea urchin eggs when we found that by using high sperm-egg ratios, untreated *Strongylocentrotus purpuratus* eggs could be made highly polyspermic. In conditions in which many sperm enter each egg, we found no change in the frequency of sperm-egg fusions after fusion of the first sperm. We conclude that there is no fast block to polyspermy in *S. purpuratus*.

S. purpuratus gametes were shed after injection of 0.5 M KCl into the body cavity. Egg jelly was removed by swirling eggs in acid seawater (adjusted to pH 5.0 with 0.1 N HCl) for 2 min. The egg suspension was then adjusted to pH 8.0 with 1 M Tris-seawater pH 8.0, and washed extensively with filtered seawater. Suspensions of eggs were inseminated and incubated before

Fig. 1 Induction of polyspermy by increasing sperm-egg ratios. Samples (1 ml) of *S. purpuratus* eggs (0.002-2.0%) were inseminated with 0.2 ml of diluted sperm. Sperm-egg suspensions were incubated for 12.5 min at 16 °C before fixation in 95% ethanol-glacial acetic acid (3:1) for 18 h with three changes of solution. Eggs were cleared in 45% acetic acid and decondensed sperm nuclei observed by phase contrast microscopy. Sperm nuclei were counted in 40-50 randomly selected eggs. Final sperm densities: $\bullet$, 4×10^8 ; $\circ$, 4×10^8 ; $\square$, 4×10^7 ; $\blacksquare$, 4×10^6 ; $\triangle$, 4×10^5 sperm per ml.



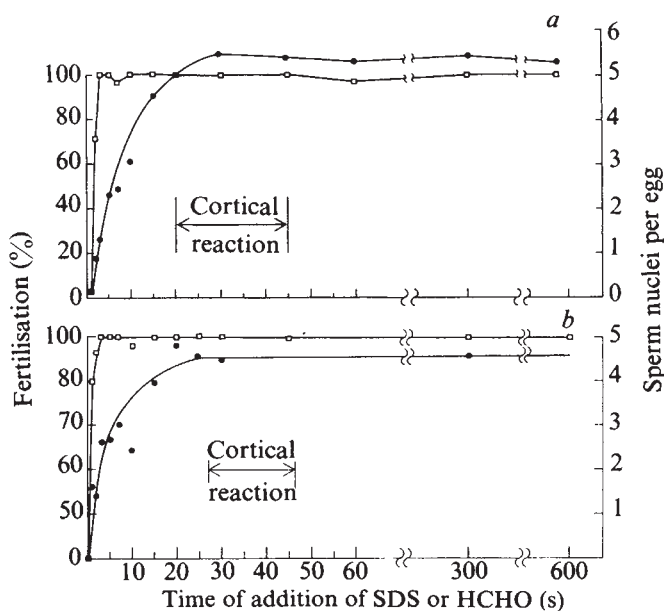


Fig. 2 Time course of sperm-egg fusions. *a*, samples (1 ml) of eggs were inseminated at time 0 by addition of 0.2 ml of sperm (8×10^7 per ml final). Eggs and sperm from the same sources were used throughout. Supernumerary sperm were killed by addition of 1 ml of 0.0075% SDS in seawater. This suspension was diluted with 4 ml of seawater 15 s later. Eggs were allowed to incubate before fixation as described in Fig. 1. Fertilisation was scored positive if a male pronucleus was visible within the egg. *b*, HCHO (0.037%) was substituted for SDS. All other procedures are the same as in *a*. $\square$, % fertilised; $\bullet$, number of sperm nuclei per egg (average value).

fixation. Eggs were cleared and decondensed sperm nuclei inside the eggs were counted using phase microscopy (Fig. 1).

We found that 100% polyspermy, with up to six sperm nuclei per egg, occurred in untreated *S. purpuratus* eggs fertilised at sperm-egg ratios greater than 10^4 (Fig. 1). This high level of polyspermy is normally achieved only by the use of polyspermy-inducing agents, such as nicotine or soy bean trypsin inhibitor. We believe our observations with untreated eggs result from the use of very high sperm-egg ratios, while most workers use ratios in the range 10^2 – 10^4 sperm per egg^{6,7}.

The observation that *S. purpuratus* eggs can become highly polyspermic suggests either (1) that at high enough sperm concentrations multiple sperm-egg fusions can occur before a fast block is established 2–3 s after insemination, or (2) that there is no complete fast block and sperm continue to fuse until the cortical block is established. To test these alternatives we determined when additional sperm were fusing with the egg in conditions which produced high levels of polyspermy. We added the spermicidal agent sodium dodecyl sulphate (SDS) or formaldehyde (HCHO) to a sperm-egg mixture at various times after insemination, to measure the number of fusion events which had occurred before their addition⁸. The addition of spermicidal agents at these times apparently prevents further sperm-egg fusion, but allows those sperm which have already fused to penetrate the egg and undergo nuclear decondensation. Accordingly we define sperm-egg fusion as an event which is a direct precursor of sperm penetration and nuclear decondensation, and which renders these subsequent processes insensitive to SDS or HCHO. Although we do not know the nature of the event which makes sperm penetration insensitive to SDS or HCHO, it is not simply binding of sperm to the egg surface, since hundreds of sperm bind at high sperm egg ratios before the addition of spermicidal agents, but only a few subsequently enter the egg. It seems reasonable that the event which makes sperm penetration insensitive to SDS or HCHO is actual fusion of the sperm and egg plasma membranes.

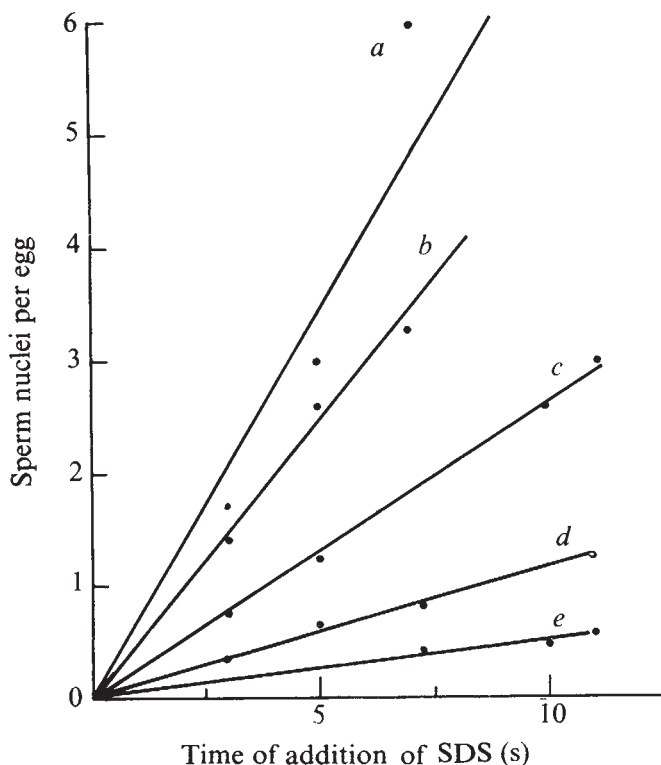
The timing of sperm-egg fusions was assayed by adding

0.0075% SDS or 0.037% HCHO to a sperm-egg suspension at various times after the gametes were mixed, and diluting 15 s later with the addition of 2 volumes of seawater. The eggs were fixed 12.5 min after insemination to count the decondensed sperm nuclei they contained. The concentration of spermicidal agent used was the minimum necessary to prevent fertilisation (that is, no elevation of a fertilisation membrane and no decondensed sperm nuclei) when added to eggs 1 s before sperm were added. The spermicidal agents were diluted routinely before this minimum effective concentration 15 s after their addition to limit their possible effect on the eggs. Although not done routinely, dilution could be performed at 1 s without altering the results, suggesting that SDS and HCHO are both fully effective within 1 s of their addition. These agents do not cause polyspermy by themselves, since the maximum level of polyspermy attained when they were present was no more than in control eggs not exposed to SDS or HCHO.

Figure 2 represents the results of a typical experiment to determine the time of occurrence of SDS or HCHO-sensitive sperm-egg fusions at sperm-to-egg ratios which produced polyspermy. A hundred per cent fertilisation, as judged by the subsequent appearance of at least one decondensed nucleus per egg, was achieved by 3–5 s after insemination; yet sperm-egg fusion continued for 25 s more. We conclude that fusion of the first sperm does not trigger an absolute block to the fusion of additional sperm. Our results support the concept of an absolute block to subsequent sperm entry which occurs at the time of the cortical reaction, since no additional fusion events occur after this time.

Although our results rule out a complete fast block to further sperm entry, there might have been a partial fast block (that is, a reduction in the rate of sperm-egg fusions after fusion of the first sperm) which the data in Fig. 2 do not rule out. If there were a partial fast block, the rate of sperm-egg fusions, as measured in a population of eggs, would decrease after 100%

Fig. 3 The time course of sperm-egg fusions, determined over a wide range of dilutions of a single sperm suspension. At the indicated times supernumerary sperm were killed by addition of SDS and the eggs were processed as under Fig. 2. Final sperm densities: *a*, 5×10^7 ; *b*, 2.5×10^7 ; *c*, 1.25×10^7 ; *d*, 5×10^6 ; *e*, 2.5×10^6 per ml.



fertilisation had been achieved, due to the lowered receptivity of the egg surface triggered by the first sperm. In the absence of any fast block, sperm-egg fusions would occur at the same rate before and after 100% fertilisation. The consistent result of experiments to test these alternatives critically is presented in Fig. 3. The rate of sperm-egg fusions was linear with time and proportional to the sperm-egg ratio. Moreover, in each case, the rate was identical before and after 100% fertilisation. We conclude that there is no change in egg receptivity to further sperm for at least 12 s after fusion of the first sperm, or no fast block.

Our conclusion that there is no fast block presents a paradox. As stated earlier, there are hundreds of sperm binding to the egg surface between the time of insemination and the complete cortical block. Without a fast block, how are all but a few of these supernumerary sperm excluded during this interval? We propose that the egg surface and surface coats act as filters to limit the fusion of these additional sperm. One such filter is the jelly coat of the egg, as proposed by Runnstrom and Manelli⁷. We have also found that in *S. purpuratus* eggs the intact jelly coat reduces the level of polyspermy obtained at moderate sperm-egg ratios below the level for eggs without a jelly coat. Even with the jelly layer removed, however, the number of bound sperm which can enter the egg is severely limited. The additional barrier may be a property of the egg's vitelline layer or the egg plasma membrane, which provide a limited number of effective sites or a time-delay barrier for sperm-egg fusion.

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Factors released from sea urchin eggs affect cyclic nucleotide metabolism in sperm

Cyclic nucleotide phosphodiesterase inhibitors and exogenous cyclic AMP or cyclic GMP can increase or maintain mammalian sperm motility, respiration rate and fructolytic rate¹⁻⁷. Sperm from various species have been shown to contain high activities of cyclic AMP-dependent protein kinase⁸⁻¹⁰, guanylate cyclase^{11,12}, cyclic nucleotide phosphodiesterase¹¹, phosphoprotein phosphatase¹³, and adenylate cyclase^{11,14}. These and other observations have led to speculation that cyclic nucleotides regulate or modulate sperm motility and metabolism¹⁵, and that this modulation may occur during the processes of capacitation or fertilisation¹⁵⁻¹⁷. The latter hypothesis requires that factors from the female or from the ovum itself alter sperm cyclic nucleotide levels. Until now there has been no demonstration of a naturally occurring factor of animal origin that can substantially change cyclic nucleotide levels in sperm¹⁵. The purpose of this study was to determine whether or not there are factors released from eggs that can alter sperm cyclic nucleotide levels. Gametes from sea urchins were used because both eggs and sperm can be obtained in large quantities, and

because factors released from sea urchin eggs have been known for some years to alter sea urchin sperm motility¹⁸⁻²⁰ and respiration^{20,21}.

Eggs and sperm were collected and washed as described previously²². Factors released from eggs (FRE) were obtained from the sea urchins, *Strongylocentrotus purpuratus* or *Lytechinus pictus*, by allowing eggs (10-20 mg wet weight ml⁻¹) to stand in a medium of simulated seawater²² for various times at 15-17 °C. The supernatant fluid above the eggs ('egg water') was collected by gentle centrifugation in a clinical centrifuge at 15-17 °C. The supernatant fluid containing FRE was added to incubation tubes containing artificial seawater with or without theophylline. To start the incubation (15-17 °C), 0.25 ml sperm suspended in artificial seawater (40-80 mg wet weight ml⁻¹) was added to each incubation tube. The incubations were terminated by addition of 1.0 ml 0.3 N perchloric acid containing traces of cyclic ³H-AMP and cyclic ³H-GMP to monitor recoveries. Cyclic AMP and cyclic GMP were purified and assayed as described elsewhere²³⁻²⁷.

Cyclic AMP concentrations in *S. purpuratus* sperm were increased approximately twofold by 1.5 mM theophylline after 1-min incubation (Table 1). FRE alone increased sperm cyclic AMP concentrations by about sevenfold, and the combination of FRE plus theophylline increased cyclic AMP concentration 100-fold. On the other hand, cyclic GMP in *S. purpuratus* sperm was decreased by about 40% by FRE. In contrast to their effects on cyclic AMP concentrations, theophylline plus FRE did not exert synergistic effects on cyclic GMP concentrations. Effects similar to those observed with FRE plus theophylline were observed with FRE plus 1-methyl-3-isobutylxanthine, another phosphodiesterase inhibitor.

Table 1 Effect of FRE from *S. purpuratus* on cyclic nucleotides in *S. purpuratus* sperm

FRE	Theophylline	Cyclic AMP (nmol per g wet weight sperm)	Cyclic GMP
None	None	2.5±0.7	0.76±0.12
None	1.5 mM	4.7±0.7	1.0±0.16
0.1 ml	None	16.6±2.0	0.46±0.04
0.1 ml	1.5 mM	267±26	1.13±0.16

Number of observations six; values represent mean ± s.e.m.

All sperm incubations were for 1 min at 15-17 °C.

Time zero cyclic AMP concentrations were 2.9±0.9 (*n* = 12) and those for cyclic GMP were 0.83±0.13 (*n* = 12) nmol per g wet weight. Time zero values were obtained by the addition of 0.3 N perchloric acid to incubation mixture before addition of the sperm.

Synergistic effects of FRE and theophylline on cyclic AMP content were also observed in sperm from *L. pictus*, although the changes were of smaller magnitude than in *S. purpuratus* sperm (Table 2). FRE from either *L. pictus* or *S. purpuratus* eggs in combination with theophylline increased cyclic AMP more than theophylline alone. Unlike the situation with *S. purpuratus* sperm, however, FRE alone did not increase cyclic AMP in *L. pictus* sperm (Table 2).

In addition to being increased by FRE and theophylline, cyclic AMP concentrations in *L. pictus* sperm were also increased by diluting the sperm in simulated seawater. Dilution of the sperm in the incubation mixture increased sperm cyclic AMP by about twofold by 1 min after dilution (Table 2). The increase was transient; by 10 min after dilution, concentrations were equal to or less than those at time zero. Such an effect of dilution was not seen with *S. purpuratus* sperm.

Because of the greater FRE-induced changes in cyclic AMP, *S. purpuratus* sperm were used in studies designed to partially characterise the cyclic AMP-elevating factor(s) present in FRE. The cyclic AMP response to increasing amounts of FRE in the presence of 1.5 mM theophylline seemed to be hyperbolic (data not shown). The same batch of FRE that was used to obtain the data in Table 1 caused a half-maximal increase in sperm cyclic AMP when about 10 µl were added to the incuba-

Table 2 Effects of FRE from *L. pictus* and of *S. purpuratus* on cyclic AMP in *L. pictus* sperm

	Theophylline (mM)	Cyclic AMP (nmol per g wet weight sperm)		
		1 min	10 min	30 min
No FRE	0	9.3±1.1	2.4±0.3	1.3±0.01
	0.15	13.4±1.1	3.4±0.3	—
	0.38	16.7±1.5	3.8±0.5	—
	1.5	21.1±1.6	5.1±1.4	3.8±0.6
<i>L. pictus</i> FRE (0.1 ml)	0	10.2±0.4	3.1±0.2	1.6±0.06
	0.15	22.4±1.7	4.9±0.3	—
	0.38	27.6±2.4	6.3±0.4	—
	1.5	40.5±3.7	9.6±0.8	5.8±0.2
<i>S. purpuratus</i> FRE (0.1 ml)	0	9.9±0.5	3.2±0.5	1.2±0.1
	0.15	17.3±0.9	4.0±0.2	—
	0.38	23.9±1.5	5.5±0.3	—
	1.5	35.0±2.2	8.3±0.4	5.5±0.8

Values represent mean ± s.e.m. of six observations at 1 and 10 min and of four observations at 30 min.

Time zero (see Table 1) cyclic AMP level in these sperm was 4.1 ± 2.1 (n = 11).

tion mixture. No increases were detected in cyclic GMP with up to 200 µl FRE in the presence of theophylline.

When a saturating amount of FRE was tested, there was some loss of cyclic AMP-elevating activity after dialysis, complete loss after ashing, but no significant loss after boiling FRE for 30 min. Two peaks of cyclic AMP-elevating activity were detected after filtration of 10 ml FRE on Sephadex G-50 columns (2.6 × 34 cm) equilibrated with simulated seawater. One of these peaks migrated in the void volume and was non-dialysable, whereas the other was retained by the column.

The mechanism of the FRE-induced elevation in cyclic AMP and reduction in cyclic GMP remains to be clarified. Direct stimulation of sperm adenylate cyclase by FRE in broken cell preparations has not been demonstrable, but extensive variations in assay conditions have not been examined. On the other hand, a potent inhibitor of sea urchin sperm guanylate cyclase in cell-free systems has been detected as a component of FRE

Table 3 Inhibition of sea urchin sperm guanylate cyclase by FRE

FRE (µl)	Guanylate cyclase activity (pmol cyclic GMP formed min ⁻¹)
0	744
5	527
10	409
25	299
50	263
100	211

Assay mixture contained 19.2 mM sodium azide, 12 mM theophylline, 0.45 mM GTP, 0.9 mM MnCl₂, 5 × 10⁵ c.p.m. ³H-GTP (5.6 Ci mmol⁻¹), the indicated amounts of FRE, 30 mM triethanolamine buffer at pH 7.8, and washed particles from 0.5 mg (wet weight) of sea urchin sperm²². The cyclic ³H-GMP formed during 15 min at 30 °C was determined as described elsewhere¹².

(Table 3). This material is stable to boiling, non-dialysable, and destroyed by ashing. The inhibitor is not adsorbed by charcoal and is not extracted from the simulated seawater by benzene or ether. Its inhibitory potency is much greater at low than at high sperm protein concentrations. Whether or not the inhibitor has any selectivity for guanylate cyclase over other enzymes, and whether or not it is the same as the non-dialysable substance(s) that increase cyclic AMP concentrations is not yet known.

These data constitute the first demonstration of alterations of sperm cyclic nucleotide concentrations by possible natural effectors. The potencies of the substance(s) that inhibit guanylate cyclase and increase cyclic AMP concentrations in sperm from sea urchins raise the possibility of their physiological importance in regulating sperm motility and metabolism. The possible release of similar factors from eggs of other species should be explored.

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Taxis to a conjugation-inducing substance in the ciliate *Blepharisma*

For conjugation of *Blepharisma intermedium* to take place interaction of soluble gamones with the cells is essential for union between complementary mating types I and II (refs 1 and 2). Type I cells excrete gamone 1 which transforms type II cells so that they can unite. Type II cells excrete gamone 2 which similarly transforms type I cells. Moreover, each gamone promotes the production of the other. Gamone 1, blepharmane, is a glycoprotein of molecular weight 20,000 (refs 3 and 4). Gamone 2, blepharismone, is calcium 3-(2'-formylamino-5'-hydroxybenzoyl) lactate⁵.

In a preliminary observation, in which a capillary containing gamone was placed in a cell suspension, type I cells were attracted by gamone 2. The observation has been confirmed here by the improved method using a microscope slide and coverslip (Fig. 1).

Clones A₁ (albino), N, D₃s and 4a of *B. intermedium* were used. A₁ and N were mating type I and D₃s and 4a were mating type II. The general techniques for the culture and handling of cells have been described elsewhere¹. Unless specified, cells were washed with and suspended in buffer¹ and starved for at least 1 d before use. Gamone 1 was purified as indicated³. Synthetic gamone 2 was supplied by Dr T. Tokoroyama, Osaka City University, and purified before use^{5,6}. One unit of gamone activity was defined as the smallest amount of gamone activity that can induce homotypic cell union in 500 cells suspended in 1 ml buffer¹. It corresponds to activities of 0.06 and 1.0 ng of purified gamones 1 and 2, respectively.

The taxis value was defined as described in Table 1 and the semi-quantitative measurement showed that type I cells were

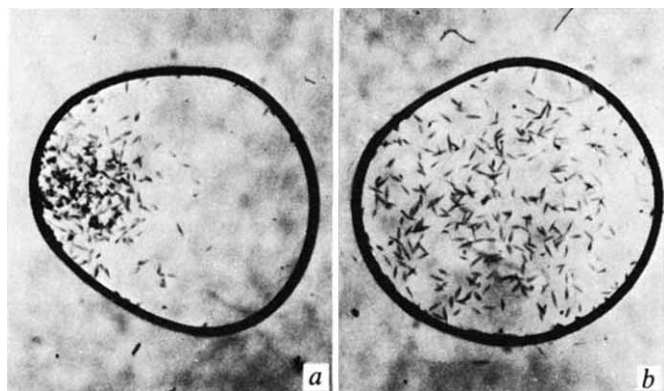


Fig. 1 Taxis of type I (A_1) cells to gamone 2. 10 μ l of type I (A_1) cell suspension and 1 μ l of cell-free fluid of the same cell suspension containing gamone 2 of 1,000 $U\ ml^{-1}$ (a) or none (b) were placed on a microscope slide and covered by a coverslip lifted 0.16 mm by spacers. The two drops were arranged so that, after covering, the smaller, satellite drop was situated very close to the left side of the larger, cell-containing drop. The two drops were then merged together by slightly moving the coverslip. Photographed after 30 min. Diameters of the disks were approximately 5 mm.

attracted by gamone 2 but not by gamone 1, while type II cells were attracted by neither (Table 1). The results were always reproduced in at least duplicate experiments.

Taxis values of type I (A_1) cells to different concentrations of gamone 2 are shown in Fig. 2. The threshold concentration of gamone 2 for the attraction may be estimated to be 10^{-6} $U\ ml^{-1}$ or 10^{-6} $ng\ ml^{-1}$. The sensitivity of type I cells to gamone 2 is, therefore, much higher in taxis than in the cell union.

Well-fed cells which are known not to respond to gamone 2 by cell union (H.H. and A.M., unpublished) were also not attracted by gamone 2. When such cells were washed, suspended in the buffer and observed during 9 h starvation, the taxis value suddenly increased between the third and the fourth hour reaching a maximum at the fourth hour (Fig. 3). Cell union also appeared after 4 h (Fig. 3). As the induction of cell union took 1 h in starved A_1 cells, the results indicate that the ability of cells to respond to gamone 2 by taxis and by cell union appears at nearly the same time, after 3 h of starvation.

The treatment of A_1 cells by cycloheximide (20 $\mu g\ ml^{-1}$, 2.5 h) did not change the ability of cells to be attracted by gamone 2. This treatment completely inhibits protein synthesis and the formation of cell union, which requires protein synthesis⁷ (H.H. and A.M., unpublished), suggesting that cell union and

Fig. 2 Taxis values of type I (A_1) cells to different concentrations of gamone 2.

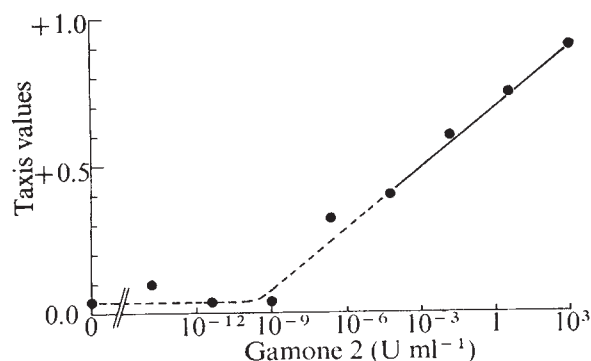


Table 1 Taxis values of mating types I and II of *B. intermedium* to their gamones

Attractants	Cells			
	Mating type I A_1	N	Mating type II $4a$	D_{3s}
Gamone 1	-0.03	0.00	0.00	0.06 0.03*
Gamone 2	0.84	0.61	0.01	-0.03
Control (cell-free fluid)	-0.01	0.02	0.01	-0.01

Taxis values were obtained by the similar apparatus in Fig. 1, using gamones 1 and 2 of 4,000 and 1,000 $U\ ml^{-1}$ respectively except the one marked by * in which gamone 1 was 130 $U\ ml^{-1}$. After 30 min from mergence of two drops, the cells were counted in each half of the drop parted evenly by a plane perpendicular to the line which passes through the centre of the cell-containing drop and the merging point of the two drops. The attraction of cells was expressed by the taxis value, $(a-b)/(a+b)$, where a and b were the number of cells in one half containing the merging point and the number of cells in the other half. Each value was the average of the values obtained by four experiments in which the relative position of the drops was changed by rotating the axis of the cell-containing drop, which was perpendicular to the surface of the coverslip, by 0° , 90° , 180° and 270° respectively.

taxis are independent. Whether gamone 2 receptors are different in these two responses is still to be determined.

Positive taxis of ciliates to the complementary mating type or its specific product has been reported only in *Tetrahymena*. In this sessile ciliate, complementary mating types, if placed within a certain distance, orient and stretch toward each other (T. M. Sonneborn, personal communication, cited in ref. 2). In view of the obvious adaptive value of a sexual attractant

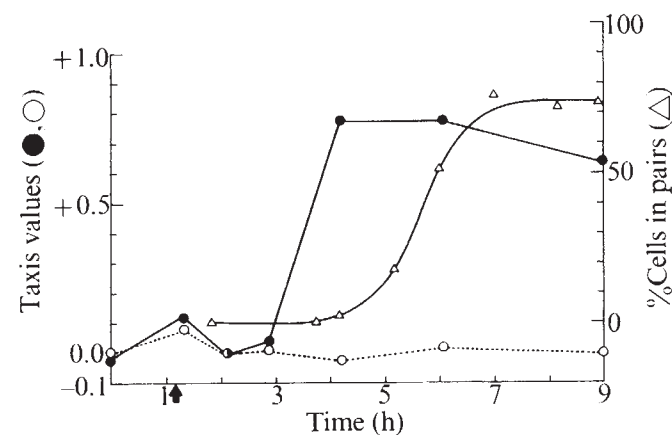


Fig. 3 Gamone taxis and gamone-induced cell union during starvation of well-fed type I (A_1) cells. Taxis values were measured on samples taken at different times during starvation. United cells were observed on a sample to which gamone 2 was added (800 $U\ ml^{-1}$) at the time indicated by the arrow. ●, Taxis to the cell-free fluid with gamone 2 (1,000 $U\ ml^{-1}$); ○, taxis to the cell-free fluid without gamone 2; △, percentage of cell number in united cells.

and its general occurrence in eukaryotes, it is likely that more examples of mating type specific attraction will be found in ciliates.

The development of gamone taxis only in type I cells of *B. intermedium* may be compared with the fact that in many organisms only one of the two sexes attracts the other by a pheromone. The finding that type I cells respond to extremely low concentrations of gamone 2 demonstrates that ciliates have a highly sensitive sensory mechanism to specific molecules,

and thus introduces a new system of chemoreception amenable to molecular analysis.

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Biogenesis of chemotactic molecules by the arachidonate lipoxygenase system of platelets

BLOOD platelets contain two aggregation-activated enzyme systems that oxygenate arachidonic acid. One system is a cyclo-oxygenase that converts arachidonic acid into the biologically active endoperoxide prostaglandin G_2 (PGG_2), a precursor of classical prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$), as well as several non-prostanoid hydroxy fatty acids¹. The second aggregation-activated platelet enzyme is a lipoxygenase that transforms arachidonic acid into 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE)².

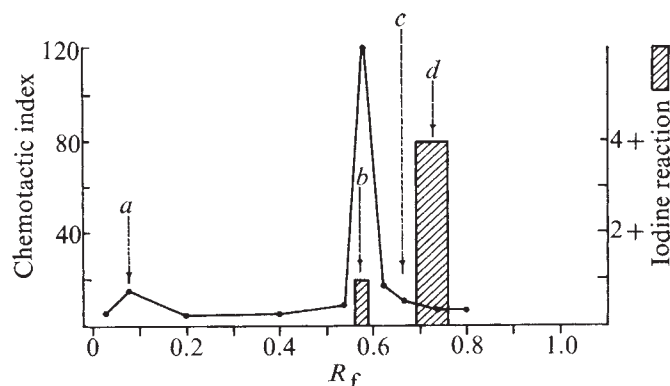


Fig. 1 TLC of the ether-extractable products obtained after incubation of 100 μ g of arachidonic acid with soluble platelet lipoxygenase (from 40 ml of human blood). Incubation was conducted in 0.1 M Tris-HCl/0.1 M K_2HPO_4 buffer (pH 8.2) at 30 °C for 90 min. Total reaction volume was 1 ml. Plates of silica gel 60 (Merck) (activated at 110 °C for 2 h) were developed to 15 cm with isopropyl ether-ethyl acetate-acetic acid (80:20:3 v/v). Chemotactic activity was assayed for one-fifth of each indicated fraction of the chromatogram and is expressed as a chemotactic index $CI = (C_a - C_0)/(C_b - C_0)$, where C_a , C_b , and C_0 are the average number of polymorphonuclear leukocyte indicator cells per $\times 430$ field attracted by the test agent (C_a), positive control: cell-free *Escherichia coli* growth medium (C_b), and buffer medium (C_0). Chemotaxis assays were performed in duplicate and five fields were counted and averaged for each assay. Chemotactic activity was primarily associated with the iodine-positive band at R_f 0.57 which was subsequently identified as HETE (see text). The concentration of HETE subjected to chemotaxis measurements was approximately 1 μ g ml^{-1} as estimated by GLC. Arrows indicate R_f values for lipid standards, a, PGE_1 , PGE_2 (R_f 0.07); b, ricinoleic acid (R_f 0.57); c, arachidonyl alcohol (R_f 0.66); d, arachidonic acid (R_f 0.72).

Although HETE is produced in significant quantities by aggregated platelets, no biological function for this compound has been reported. We now have evidence that HETE is a potent mediator of neutrophil chemotaxis.

We suggested³ that oxygenated arachidonate products may be the chemotactic mediators released by aggregated blood platelets⁴ because identical conditions are required for both arachidonate oxygenation and the generation of chemotactic activity^{2,4}. Since we had demonstrated that photo-oxidised arachidonic acid is chemotactic for neutrophils⁵, this hypothesis seemed even more tenable.

To test this hypothesis, chemotactic factor was generated by incubating the soluble lipoxygenase fraction² of human blood platelets with pure arachidonic acid. The chemotactic activity of the reaction mixture and its subsequent fractions was measured as before⁵, using non-autologous human polymorphonuclear leukocytes as indicator cells. After incubation, the products were acidified (pH 4) with 2 M citric acid and extracted into diethyl ether². All chemotactic activity was extracted into the ether phase, which was dried under nitrogen, resuspended in methanol, and subjected to thin-layer chromatography (TLC). All bands that could be visualised with the aid of 254-nm radiation, iodine vapour, and ninhydrin were removed from the chromatogram and tested for chemotactic activity.

As Fig. 1 shows, chemotactic activity was almost exclusively associated with an iodine-positive band at R_f 0.57. This material, evident as a dark absorption band under 254-nm illumination, exhibited no reaction with ninhydrin. The chemotactic band was clearly separated from authentic samples of PGE_1 , PGE_2 , arachidonyl alcohol, and arachidonic acid, but comigrated with ricinoleic acid. An iodine-positive band corresponding to unreacted arachidonic acid was also detected on the chromatogram. Another incubation, identical to the experiment described in Fig. 1 except for the addition of indomethacin (1 μ g ml^{-1}), gave a chemotactic profile almost identical to that shown in Fig. 1. This result indicated that the reactions studied were not due to the action of the platelet cyclo-oxygenase system which is inhibited by indomethacin at these concentrations¹. Other incubations which omitted the lipoxygenase or included lipoxygenase boiled for 30 min were not chemotactic and did not yield the R_f 0.57 band. These data suggested that platelet lipoxygenase was converting arachidonic acid into a chemotactic hydroxy fatty acid, probably HETE.

The presence of HETE was indicated by gas-liquid chromatography (GLC) (3% OV-225 on 120/100 Chromasorb WAW with column temperature 220 °C) of the active compound after catalytic reduction (H_2 -Pd on barium sulphate) and methylation (ethereal diazomethane). A single elution peak was observed which had the retention time of the methyl ester of a 20-carbon hydroxy fatty acid. The identity of the chemotactic material as HETE was confirmed by GLC-mass spectrometry (MS). A mass spectrometer (Finnigan 3300) fitted with a column of 1% OV-17 was programmed from 160–240 °C at 8 °C per min to obtain an electron impact fragmentation pattern of the reduced, methylated unknown. Prominent peaks at m/e 229, 200 and 197 were obtained which corresponded precisely to the reported mass spectrum of reduced, methylated HETE². The molecular ion ($M=342$) was absent.

To examine further the conditions required for the synthesis of chemotactic mediators, a series of incubations of platelets and platelet components with arachidonic acid was evaluated for chemotactic products using the same protocol as in Fig. 1. As Table 1 shows, chemotactic activity was always associated with the occurrence of an I_2 -positive TLC band at R_f 0.57 which had the GLC properties of a 20-carbon hydroxy fatty acid, indicating that HETE was the only chemotactic mediator elaborated by platelets.

Table 1 also indicates that both arachidonic acid and platelet-free serum function as prochemotactic substrates

Table 1 Reaction matrix for incubations conducted as described in Fig. 1

	Arachidonic acid (100 µg)	LPS (1 mg)	Platelet-free plasma (100 µl)
Whole platelets	+	+	+
Soluble lipoxigenase	+	—	+
Platelet acid extract	+	ND	+
Arachidonic acid, 100 µg	—	—	—
<i>S. typhosa</i> lipopolysaccharide 1 mg	—	—	—

The products of each incubation were worked up and assayed for chemotactic activity as outlined in Fig. 1 and the text. + indicates that HETE was the major chemotactic product as evidenced by TLC and GLC analysis. — denotes the absence of chemotactic products. Whole platelets², soluble lipoxigenase³, and platelet sulphuric acid extract⁶ were prepared from 40 ml of blood (0.1 mg platelet protein) according to the referenced procedures. Platelet-free plasma (PFP) was obtained by differential centrifugation of human blood⁴. Indicated quantities of arachidonic acid, LPS and PFP are for 1-ml incubation volumes. ND, not determined.

for any of the platelet fractions. This circumstance raises the possibility that arachidonic acid is the prochemotactic factor observed⁴ in platelet-free plasma. Furthermore, the failure of *Salmonella typhosa* lipopolysaccharide (LPS) to generate chemotactic activity with soluble lipoxigenase suggests that LPS-generated chemotactic activity requires both lipoxigenase and arachidonic acid as found in platelet membranes, for example, whole platelets × LPS (Table 1).

HETE was also identified by TLC and GLC-MS as a component of the chemotactic material generated by ultra-violet photo-oxidation of arachidonic acid⁵. This observation indicates that non-enzymatic oxidation of arachidonic acid can generate a biological chemotactic mediator without intervention of either platelet lipoxigenase or the complement immune system. The status of complement components in chemotaxis is still an open question, especially in view of the observation that a crystalline preparation of the presumably chemotactic C5a was not chemotactic for neutrophils or macrophages⁶.

On the basis of our results, we propose that the platelet lipoxigenase system functions in a complementary fashion with the cyclo-oxygenase system to provide chemotactic, vasoactive, and platelet-aggregating factors during haemostasis. The likelihood of other biological activities for products of the platelet endoperoxidase and hydroperoxidase seems to be great and merits further investigation.

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Thymus-like lymph node in nude mice

PANTELOURIS has reported¹ that 1 out of 60 autopsied nude mice possessed a suspected normal thymus, but histological examination was not possible. Here, we report on five nude mice with distinct lobulated thymus-like organs in the thymus area, but which can be shown to be lymph nodes, rather than thymus.

Our nude mice were maintained by mating male *nu/nu* homozygotes with female *nu/+* heterozygotes (of outbred Swiss mouse background) at the veterinary Resources Branch, National Institutes of Health, Bethesda, Maryland. We have seen previously white lobulated lymphoid tissues, whose overall appearance is that of a 'normal thymus', in 5 out of 74 nude mice of 3–5 months of age. These mice were experimentally infected with either *Trichinella spiralis* or *Schistosoma mansoni* and were housed in specific pathogen-free conditions. Histologically these thymus-like tissues were lacking in Hassall's cor-

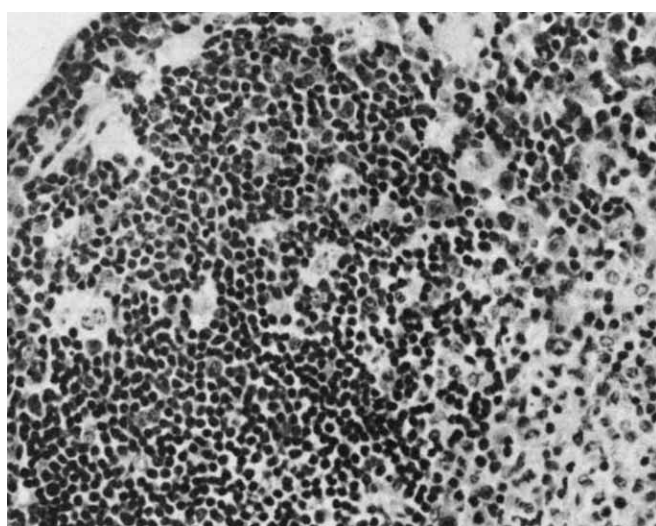


Fig. 1 No Hassall's corpuscles or thymic epithelial cells, but proliferative lymphocytes and reticuloendothelial cells were seen in this tissue (haematoxylin and eosin stain, × 300).

puscles and thymic epithelial cells. Active proliferation of lymphocytes, histocytes, and reticuloendothelial cells was evident.

In the cortex and medulla (Fig. 1) lymphoid follicles medullary sinuses and numerous plasma cells were also observed. These observations suggest that these tissues are active lymph nodes rather than normal thymus.

We have no real evidence to suggest that it is the pre-lymphoid thymic rudiment that is repopulated by the antigen-activated lymphocytes and histocytes, although this remains a possibility. These lymph nodes may be simply the anterior mediastinal or tracheobronchial lymph nodes that are greatly enlarged due to the proliferated immunolymphocytes and reticuloendothelial cells, resulting from constant antigenic stimulation.

If the nude mice were born with a genuine thymus, one would expect to see the presence of functional T cells. Thus, spleen cells from the *Trichinella*-infected nude mice with suspected thymuses, infected and non-infected *nu/+* heterozygotes and typical nude mice without thymuses, were studied in response to phytohaemagglutinin (PHA), and *Salmonella typhimurium* lipopolysaccharide (LPS). These materials are generally accepted as mitogens for mouse T and B cells, respectively. The experiments were done in triplicate. No functional T cells were found, but B cells were demonstrated in the nude mice bearing suspected thymuses (Table 1). Since, however, crossing over

Table 1 Effect of infection on suspected thymus of nude mice

Mitogen	Dosage (μg)	Trichinella-infected			Non-infected normal			<i>nu/+</i> SI
		c.p.m.	<i>nu/nu</i> * SI	c.p.m.	c.p.m.	<i>nu/nu</i> SI	c.p.m.	
0	0	1,099		1,101	1,540		534	
PHA	8	692	0.64	10,129	1,405	0.92	12,327	20.08
LPS	20	35,441	32.40	48,870	77,313	50.20	2,214	41.6

*The nude mouse with suspected thymus
SI: stimulation index

might occur between the genes for athymia and hairlessness, if these are separated, we agree with Pantelouris that the experimenter should do gross necropsy and histology examinations to ensure that nude mice are really without a thymus.

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Unexpectedly rapid action of human interferon in physiological conditions

The progression of many viral infections can be affected by the administration of exogenous interferon or the production of endogenous interferon^{1,2}, the concentration of which is greatest at the site of infection^{3,4}. Therapy with interferon generally requires large amounts^{5,6} presumably to allow for diffusion to the site. Model studies of this phenomenon *in vitro* have usually been carried out in the non-physiological conditions of relatively low concentrations of interferon and room temperature⁷⁻⁹. To understand better the factors that govern the local protective action of interferon, we have done model experiments in tissue culture to define the time and concentration variables under strict simulation of body temperature by carrying out all manipulations in a 37 °C water bath.

Duplicate HR203 human foreskin fibroblast cells were incubated in tubes 13 × 100 mm with prewarmed human leukocyte interferon (from the Antiviral Substances Program, NIAID)¹⁰, diluted in 0.5 ml Eagle's medium containing 2% foetal bovine serum. At various times the interferon was removed, and the cultures were washed four times with warm Earle's balanced salt solution and challenged with a Sindbis virus to cell input multiplicity of infection of 20:1. After 1 h for virus adsorption, cultures were washed three times and incubated for 18 h before culture fluids were assayed for viral haemagglutinin (HA).

Contrary to previous observations made in less physiological conditions⁷⁻⁹, a 30-min exposure of cells to interferon resulted in a high, although not maximal, interferon titre (Fig. 1a). Maximum titre was attained when the virus challenge was applied after 2-4 h of interferon treatment.

Shorter exposures to interferon were tried next. Figure 1b shows that a 1-min exposure resulted in almost the same interferon titre as that following 30 min of exposure. Similar results were obtained when the temperature was varied between

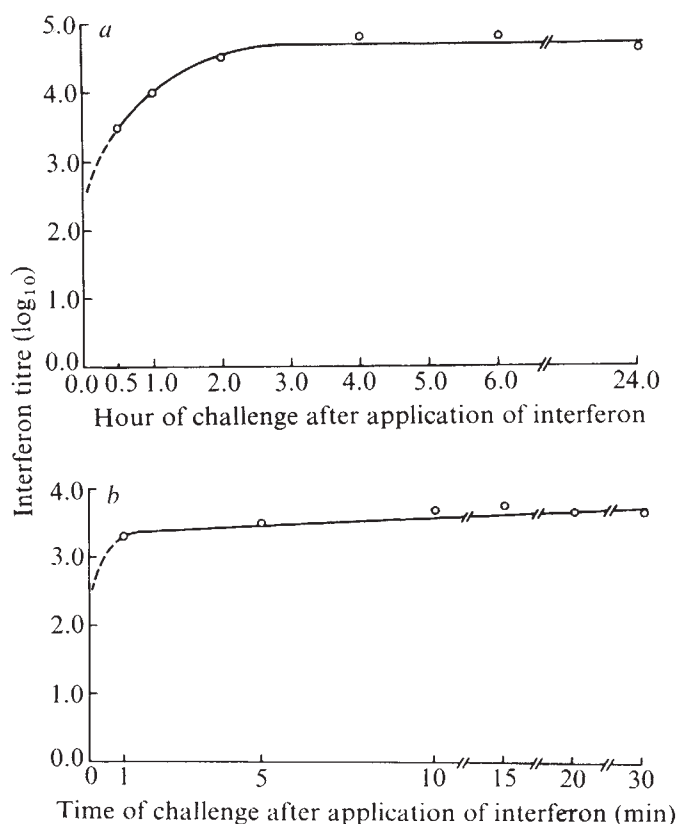
34.5 °C (temperature of upper respiratory tract) and 40 °C for a 5-min exposure.

To determine the effect of various concentrations of interferon on the level of resistance in the human cell cultures we applied either 10 U ml⁻¹, 30 U ml⁻¹ or 300 U ml⁻¹ of interferon to different cultures for various times before virus challenge. As Fig. 2 shows, the level of early antiviral activity was proportional to the concentration of interferon applied. Thus interferon at a concentration of more than 10 U ml⁻¹ was required to initiate the development of the early appearing resistance (8-16-fold inhibition) which is strong enough to be significant biologically.

Figure 2 suggests that resistance develops very slowly between 1 and 20 min of cellular reaction with interferon. Further study is required to understand the mechanism of this lag before the beginning of a second rise in cellular resistance, which leads to a higher and stable level of resistance⁸.

Several characteristics of the early developing antiviral activity suggest that it was induced by the interferon rather than by an extraneous substance. Its rapid development was species

Fig. 1 Development of interferon activity in HR203 cells challenged at various times after exposure to human interferon. The cultures were incubated with interferon dilutions for the times indicated, washed and challenged with virus. Interferon titres are expressed at the highest dilution which inhibited the virus yield threefold.



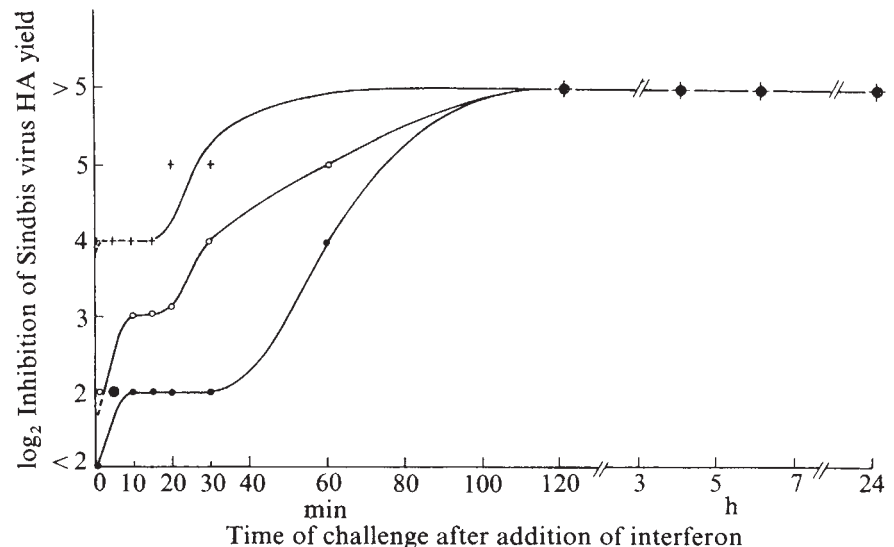


Fig. 2 Effect of interferon concentration on the level of rapidly developing resistance. Interferon was incubated with cultures for the times indicated before virus challenge. ●, 10 U ml⁻¹; ○, 30 U ml⁻¹; +, 100 U ml⁻¹.

specific, occurring in several strains of human diploid cells but not in mouse L cells. It was induced by both human leukocyte interferon and human fibroblast interferon. The same effect was observed in mouse L cells after a 5-min application of crude or semi-purified (10^7 U mg⁻¹ of protein) mouse interferon. The resistance manifested lack of viral specificity in that both vesicular stomatitis virus and the picornavirus, GD 7, were inhibited. Preincubation of the interferon with antibody to human interferon (obtained from Dr C. Anfinsen) or inhibition of RNA synthesis by actinomycin D prevented the development of early resistance.

The effect we have found is distinct from that reported when a brief exposure of cells in the cold to interferon, followed by prolonged incubation at 37 °C, was required for the development of antiviral activity¹¹⁻¹³. For example, prolonged incubation at 37 °C was not needed for the development of rapid resistance. Also, unlike the previous example, the reaction between interferon and cells at 37 °C seems to be due to direct induction of resistance. It is possible that in the cold the initial interaction of interferon with cells does not induce antiviral activity from the original binding site. The small amount of initially cell-bound interferon may simply serve as a reservoir of interferon during washing. Subsequently at 37 °C the interferon slowly elutes from the cells into the medium and then reacts with the cells to induce resistance¹⁴. In contrast, in the warm conditions we used, preliminary experiments involving antibody to interferon and repeated washings¹⁴ indicate that this type of cellular binding and subsequent elution are not required for development of rapid resistance.

Resistance to virus challenge after brief exposure to interferon does not necessarily mean that the resistance is established at the moment of virus challenge because several viruses, including Sindbis, may be inhibited when interferon is applied to cells as late as 2 h after virus^{15,16}. Nevertheless our preliminary experiments indicate that the messenger RNA for the resistance-inducing protein is transcribed as early as 30 min after a 5-min exposure of cells to 300 U interferon. Since translation of this mRNA is very rapid¹⁷ resistance probably develops after about 30 min.

To investigate whether concentrations of interferon sufficiently high for rapid effect *in vivo* occur, we estimated concentrations in the extracellular fluid around producing cells *in vivo*. We assumed that (1) a single infected cell releases its interferon into the surrounding intercellular space over a period of 6 h; (2) the half life of interferon in the extracellular fluid is similar to the rapid turnover phase in serum, that is, 10 min (ref. 3); (3) the volume of extracellular space surrounding a cell in a solid tissue varies from 12 to 120 μm^3 , and (4) that the interferon production *in vivo* may cover the same range as in cell

culture, that is 10–10,000 U ml⁻¹ in cell cultures or 0.00005–0.05 U per cell. Thus an estimated concentration of 3,000–30,000,000 U ml⁻¹ occurs in the intercellular space surrounding an interferon-producing cell *in vivo*. These levels are much more than the minimum of 10–30 U ml⁻¹ required to induce early resistance.

The biological significance of this rapid effect is clear. *In vivo*, the infected cell frequently produces both virus and interferon. A substantial lead of development of host resistance over the spreading virus would be provided by a rapid action of the interferon on surrounding cells since virus requires at least 1 h for adsorption, penetration and uncoating. This early defence would be less effective in conditions of minimal or late interferon production, low sensitivity of virus to interferon and a large volume of extracellular fluid.

The rapid action of interferon on cells has several additional implications. Is it analogous to the moderately rapid action of certain polypeptide hormones on cells^{2,18}? Also is it possible that the brief reaction induces a cellular event which, 30 min later, leads to derepression of the cistron encoded for the antiviral protein¹⁹? Study of the initial cellular event could yield information about the step(s) leading to derepression. Finally, can treatment of viral infections with interferon be improved by methods which deliver sufficient interferon to the site of infection to induce this rapid resistance?

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Immunity to Marek's disease induced by glutaraldehyde-treated cells of Marek's disease lymphoblastoid cell lines

MAREK'S disease (MD) is a common neoplastic disease of the domestic fowl caused by a herpesvirus (MDV). It is controlled in the field by vaccination with artificially attenuated strains of MDV, or more commonly with the antigenically related herpesvirus of turkeys (HVT)¹. The nature of the immune prophylaxis is not clear; protective immunity may be directed against MDV, against the development of tumours, or both. One way in which vaccination may exert its effect is by influencing the spread of MDV within the body². In contrast, infected birds may occasionally recover after showing signs of MD clinically, and in some birds MD lymphoproliferative lesions regress³, so it is likely that there is also an anti-tumour immune reaction. We have sought evidence for anti-tumour immunity in experimentally immunised birds.

MDV infection of chick kidney cells (CKCs) *in vitro* results in the expression of a number of nuclear, cytoplasmic and membrane antigens which can be visualised by immunofluorescence. Similar antigens occur on only a small proportion of MD lymphoma cells and MD lymphoblastoid line cells (about 10³ and 10 per million cells, respectively, in this study). The lymphoblastoid line cells and a variable (2–35%) proportion of MD lymphoma cells have other surface antigens which, because they occur only on cells which are thought to represent the malignant component of MD lymphomas, have been regarded as tumour specific antigens^{4,5}. The cells of lymphoblastoid lines from MD lymphomas contain the viral genome⁶, and the tumour specific antigens may be virally determined. Nevertheless it is possible to distinguish between viral antigens (as exemplified by MDV infection of CKCs) and tumour specific antigens (occurring only on non-productively infected lymphoblasts).

Lymphoblastoid line cells were used as a source of tumour specific antigens relatively uncontaminated with the known viral antigens, and CKCs infected *in vitro* with MDV were used as a source of these viral antigens. Glutaraldehyde is known to preserve or enhance the immunogenicity of antigenic structures⁷, and it was found to abolish the infectivity of MDV-infected cells in the conditions of this experiment. The induction

of tumour immunity in mice using glutaraldehyde-treated tumour cells has been reported for BALB/c tumours induced by methyl cholanthrene in BALB/c mice⁸. We have found that protection against MD can be achieved by immunisation with glutaraldehyde-treated lymphoblastoid lines as well as with similarly treated MDV-infected CKCs.

Groups of about 25 HPRS Rhode Island Red chickens were immunised (Table 1) with glutaraldehyde-inactivated tumour cells, infected lymphocytes, normal lymphocytes, lymphoblastoid cell lines or infected CKCs. A control group received no treatment, and another group was vaccinated with a standard MD vaccine (HVT). Immunised chickens were exposed to MDV at 55 d of age by mixing with birds which had been inoculated with MDV (HPRS 16 strain) 30 d previously, and which were actively shedding virulent virus. Mortality was observed until the birds were 230 d old. Birds which died from causes other than MD were eliminated from consideration. Diagnosis of MD as the cause of death was made on the basis of gross and histological visceral and neural lesions characteristic of MD⁹. The χ^2 test (with Yates's correction) was used to compare mortality in immunised groups with that of untreated birds (group F).

The results are shown in Fig. 1. As expected, birds which had been vaccinated with HVT or immunised with inactivated MDV infected CKCs were significantly protected against MD. Group G suffered a mortality of nine out of 22 ($\chi^2 = 5.54$) and group E of 5/19 ($\chi^2 = 9.96$) compared with group F (19/24). Group D, immunised with the lymphoblastoid cell lines, had a mortality of 10/23 ($\chi^2 = 4.91$). Mortality rates for the two individual cell lines were 6/13 ($\chi^2 = 2.82$) and 4/10 ($\chi^2 = 3.32$) for HPRS lines 1 and 2 respectively. The results for the two lines were consistent, and taken together the protective effect was significant at the 5% level.

The protective effect of inactivated preparations of productively infected cells, such as received by group E, is directed against virus replication or spread, as shown before^{10,11}. It is unlikely that the protection afforded by the treated lymphoblastoid cell lines was due to the viral antigens which are expressed on the small proportion of the cells which are productively infected, as the number of such cells received by group D was much less than was received by groups A and B (32- and 4-fold, respectively), which were not protected. The protective effect was probably due to the tumour specific antigens present on the cells of the lymphoblastoid cell lines. The failure of tumour cells and lymphocytes from infected chickens to protect may have been a dose effect. Malignant cells form only a minority of the cells of MD lymphomas, the remainder being inflammatory reactive cells^{4,5}. In contrast, almost 100% of the cells of MD lymphoblastoid lines carry tumour specific antigens^{4,5}. Alternatively, the tumour specific antigens present

Table 1 Immunisation schedules

Group	No. of birds*	Inoculum cell type	Age of recipients (d)		
			9	24	42
A	20	Tumour cells	21.4×10^6 †	20.8×10^6	80.6×10^6
B	21	Infected lymphocytes	82.8×10^6	76.0×10^6	75.0×10^6
C	24	Normal lymphocytes	94.8×10^6	—	83.1×10^6
D	23	Lymphoblastoid cell line cells	15.9×10^6	—	26.2×10^6
E	19	Infected CKCs	$10^{4.2}$ PFU	—	$10^{4.4}$ PFU
F	24	None	—	—	—
G	22	HVT (cell free)	$10^{3.4}$ PFU	—	—

Tumour cells were freshly explanted cells of ovarian lymphomas from chickens experimentally infected with MDV (HPRS 16 strain). Infected lymphocytes were peripheral blood lymphocytes from the same birds. Normal lymphocytes were peripheral blood lymphocytes from unexposed chickens of the same age. Lymphoblastoid cell line cells were cells of HPRS lines 1 and 2, derived originally from MD lymphomas. Because of difficulties in obtaining large quantities of these cells, 13 birds were inoculated with HPRS line 1 and 10 with line 2. In view of the similarities between the two cell lines¹², these birds were treated as a single group. Infected CKCs were chicken kidney cells infected *in vitro* with HPRS 16 strain of MDV. All cells were assayed for infectivity on CKC monolayers before inactivation by incubation at 20 °C and at a concentration of 10^8 ml⁻¹ for 15 min in 0.125% glutaraldehyde in phosphate buffered saline (PBS). After inactivation the cells were washed three times in PBS. HVT (cell free) was lyophilised herpesvirus of turkeys (strain FC 126). With the exception of group G, birds received the first inoculum in Freund's complete adjuvant, and subsequently without adjuvant.

* Birds dying of causes other than MD were removed from final group size.

† Quantities shown are doses per bird.

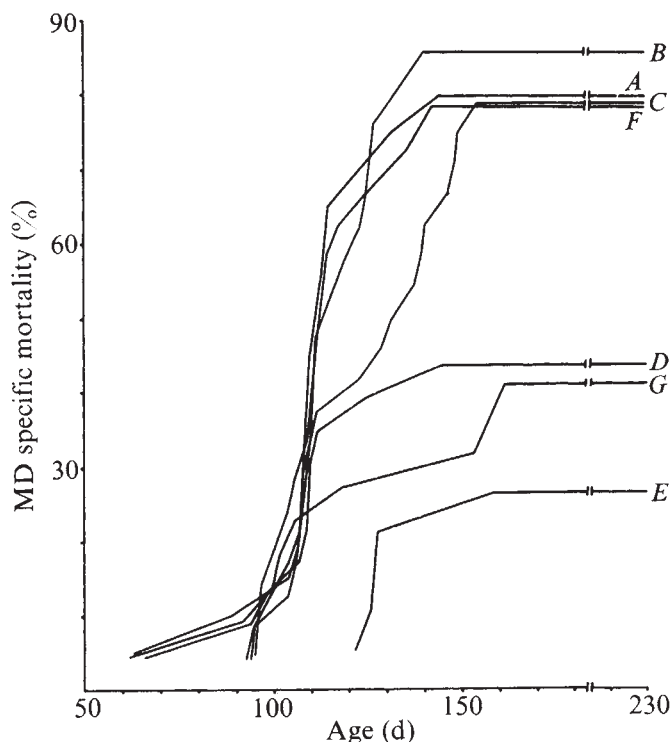


Fig. 1 Cumulative percentage of MD mortality of immunised and control chickens. Group treatments were as detailed in Table 1.

on lymphoma cells may differ from those on the lymphoblastoid cells of the cell lines, perhaps because of reaction with antibody or sensitised lymphocytes *in vivo*.

It has been suggested⁹ that resistance to MD proceeds by a two-step mechanism. The restriction of virulent virus infection brought about by vaccination may result in a much reduced incidence of malignant transformation of T lymphocytes, by reducing the probability of appropriate virus-cell interactions leading to neoplastic transformation. These transformed cells are the targets for the second step, the rejection of neoplastic cells. The immunosuppression caused by the multiplication of virus in the lymphoid organs may also exert an important influence on the outcome of infection. It is known from vaccination studies² and from experimental protection trials using inactivated virus preparations^{10,11} that immunity directed against virus specific antigens is protective. Vaccination, however, does not prevent superinfection with virulent strains of MDV, and the possibility of malignant clones arising still exists. These may be rejected by lymphocytes sensitised against tumour specific antigens. The results of our experiment show that resistance to MD may be induced with tumour specific antigens. The protective mechanism operating in birds which are resistant by virtue of vaccination, genetic composition or age may involve both these components. The nature of the tumour specific antigens and the role played by MDV in inducing their appearance remain unknown.

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Isolation of 14-nm virus-like particles from mouse brain infected with scrapie agent

SCRAPIE, a naturally occurring slow virus disease of sheep and goats, has been transmitted experimentally to the mouse, rat, gerbil, mink, hamster, vole and monkey¹. The agent has shown an unusual resistance to ultraviolet irradiation², nucleases³ and β -propiolactone⁴. The precise nature of the scrapie agent, however, has yet to be defined. It is not known whether it is a virus, some type of self-replicating cell membrane, or part of a membrane⁵. The viroid hypothesis of the scrapie agent of Diener⁶ has been discounted^{7,8}. Several different particles have been seen in thin sections of naturally and experimentally infected scrapie tissues⁹⁻¹¹. As repeated fluorocarbon extractions of mink tissues infected with Aleutian disease virus (ADV), which causes persistent infection of mink, have resulted in isolation of the virus^{12,13}, we thought it worthwhile to apply the same physico-chemical techniques in an attempt to isolate virus from mouse brains infected with scrapie agent.

The Chandler or Compton strain of mouse-adapted scrapie was obtained from Dr W. J. Hadlow¹⁴ (Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases). Four hundred, 3-week-old female Swiss albino mice of the Animal Diseases Research Institute (Western) stock were inoculated intracerebrally with 0.03 ml of a 10^{-2} mouse brain suspension of the scrapie agent. Eighteen weeks after inoculation, clinical signs of scrapie appeared in many mice and the clinical disease progressed with time. During week 20 after infection some sick mice died. Starting in the week 20 after infection, brains were collected from severely affected mice or from dead mice. At 158 d after infection, all remaining mice were killed and their brains collected. Scrapie brains (132 g in total) were homogenised in 660 ml of saline using an omnimixer at 15,000 r.p.m. for 15 min. Two parts of the homogenate were added to one part of Freon 113 (DuPont, trichlorotrifluoroethane, $\text{CCl}_2\text{F}-\text{CClF}_2$) and homogenised for 2 min at the same speed. After centrifugation of the Freon homogenate at 10,000g for 30 min, the supernatant was collected. The tissue phase was homogenised in saline and Freon extraction was repeated. Both supernatants were concentrated at 100,500g for 5 h. The ultracentrifuge supernatants were discarded and the pellets were collected. The pellets were suspended in 100 ml of saline, and Freon extraction and concentration of the supernatant were repeated. The final pellets were homogenised in 10 ml of 0.05 M Tris-HCl, pH 7.4, and labelled 'scrapie extract'. This scrapie extract contained protein (0.88 mg ml^{-1}) determined by the micro-Kjeldahl method. A 3.5-ml volume of CsCl solution (14.4 g of CsCl was added to 23.4 ml of 0.05 M Tris, pH 7.4) was put in a centrifuge tube and 1 ml of the scrapie extract was mixed with the top part of the CsCl solution. This was ultracentrifuged at 243,000g for 72 h using a Beckman swing bucket 50.1 Ti rotor. After ultracentrifugation, 10 equal fractions, each approximately 0.45 ml, were collected from the top of the tube using Pasteur pipettes. The pellet deposited in the bottom of the tube was also suspended in 0.45 ml of the Tris (fraction 11).

Two bands were visible in the CsCl gradient, one in fraction 5 and the other in fraction 7. Fractions 9, 10 and 11 were brownish in colour and appeared to contain ferritins. The buoyant

Table 1 Absorbance and electron microscopy of CsCl fractions of scrapie-infected mouse brain extract

Fraction No.	Absorbance		EM
	260 nm	280 nm	
1	0.255	0.191	No 14-nm particles
2	0.347	0.260	No 14-nm particles
3	2.561	2.376	No 14-nm particles
4	0.733	0.677	No 14-nm particles
5	2 × 2.644	2 × 2.597	No 14-nm particles
6	2.480	2.111	Some 14-nm particles
7	2.718	2.026	Numerous 14-nm particles
8	2.039	1.495	Some 14-nm particles and ferritin
9	4 × 2.034	4 × 1.178	Ferritin
10	8 × 2.530	8 × 1.309	Ferritin
11 (Pellets)	Not done		Ferritin

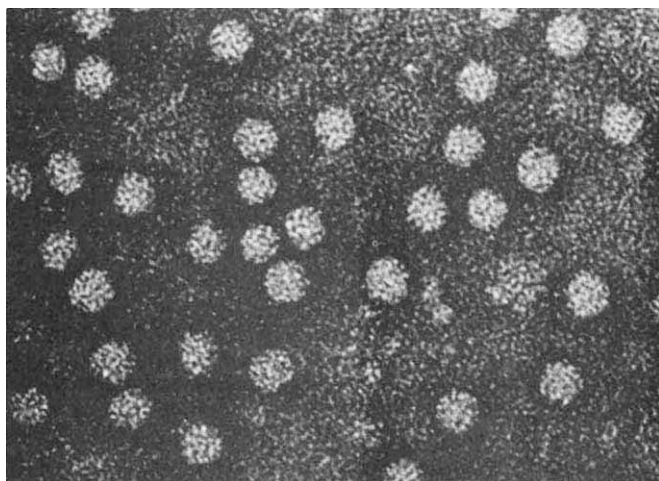
* Diluted with an equal amount of 0.05 M Tris.

densities of these two bands were determined from their refractive indices. The absorbance (*A*) of each fraction was read at 260 and 280 nm (Table 1). Then 4 ml Tris was added to each fraction and concentrated at 243,000*g* for 4 h. After ultracentrifugation, the supernatants were discarded and the pellets were resuspended in 0.4 ml of the Tris. For electron microscopy, a drop of each fraction was placed on to a Formvar membrane floating on cold distilled water and dialysed for 1 h. After this, one drop of the dialysed materials was placed on to a 400-mesh carbon coated grid, and allowed to deposit on the membrane of the grid for 3 min. The liquid on the grid was removed with a filter paper. Finally, the grid was stained with 2% potassium phosphotungstate, pH 7.2, and examined with an electron microscope (Hitachi model HU-12A).

Numerous virus-like particles, measuring about 14 nm in diameter, were observed in fraction 7 and lesser amounts were observed in fractions 6 and 8 (Fig. 1). The particles seemed to be spherical. Many were intact, but some seemed to be empty or incomplete. Fractions 8, 9, 10 and 11 contained ferritins¹³. No virus-like particles could be detected in fractions 1–5. These virus-like particles observed in the band in fraction 7 have a buoyant density 1.341–1.350 *g cm*⁻³, whereas the band observed in fraction 5 has a density of 1.296–1.305 *g cm*⁻³ in CsCl.

Similar extracts with 5-week-old normal mice brains from the same mouse colony yielded only one visible band in fraction 5, with a buoyant density of 1.296–1.305 *g cm*⁻³ in CsCl. No band was observed in fraction 7 from normal mice brain extracts. Electron microscopy of fraction 5 of the normal mice brain extracts did not reveal any particles. In the pool of fractions 6 to 8 some ferritin appeared, but no particles comparable with those seen in the scrapie infected mouse extracts.

Fig. 1 14-nm virus-like particles isolated from mouse brains infected with Chandler strain of scrapie agent (×408,000). Negatively stained with 2% potassium phosphotungstate, pH 7.2.



As known virus control, ADV was ultracentrifuged in the CsCl in the same run as a scrapie extract. The ADV was banded at a buoyant density of 1.405–1.416 *g cm*⁻³ which is typical of the parvovirus^{13,15}.

If the 14-nm particles isolated from scrapie-infected mouse brain are indeed a virus, they represent the smallest one ever reported. This size corresponds to the theoretical smallest size limit for a spherical virus¹⁶. It is not known whether these particles are indeed the scrapie agent. All the fractions shown in Table 1 are being tested by inoculation of mice to determine the amount of scrapie infectivity in each of them.

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Latent infection of the peripheral ANS with herpes simplex virus

IN both experimentally infected animals^{1,2} and in asymptomatic human subjects^{3,4} herpes simplex virus (HSV) can establish a latent infection in the sensory ganglia of the nervous system. It is probably the periodic reactivation of virus within these ganglia that gives rise to recurrent herpetic eruptions on

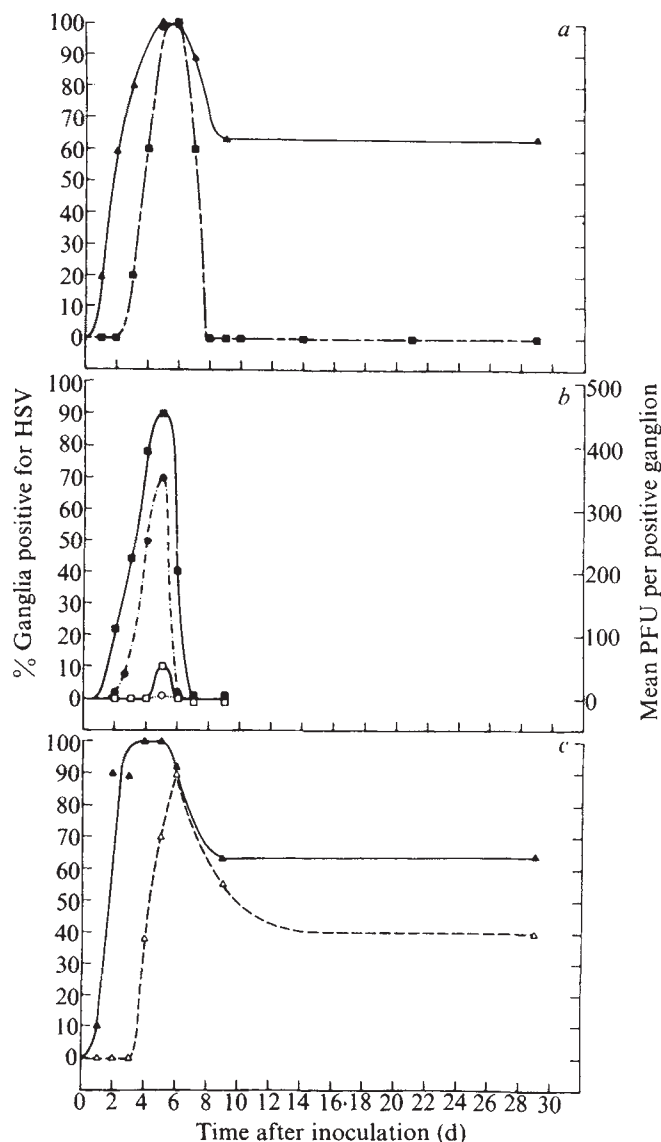


Fig. 1 Acute and latent HSV infection of the SCG after unilateral inoculation into the anterior chamber of the eye. *a*, Time course of SCG infection with HSV: the percentage of ipsilateral ganglia positive for virus by homogenisation (●) and explantation (▲) at various intervals after unilateral anterior chamber inoculation. *b*, Comparison of homogenates of ganglia ipsilateral and contralateral to the side of injection: homogenisation of ganglia at various times after unilateral anterior chamber injection presented both as the percentage of ipsilateral (●) and contralateral (○) ganglia positive for HSV and as the mean number of PFU per positive ipsilateral (●) and contralateral (○) ganglion. *c*, Comparison of explants of ganglia ipsilateral and contralateral to the side of injection: the percentage of ipsilateral (▲) and contralateral (△) ganglia positive for virus at various times after unilateral HSV injection of the anterior chamber. In all experiments the SCG of at least ten mice were assayed at each interval.

tories) were used. HSV (CHR-3 strain of type 1) was prepared and assayed on primary rabbit kidney (PRK) cells as described previously¹⁰. The SCG, which is situated near the bifurcation of the common carotid artery, was removed under a dissecting microscope in sterile conditions, washed three times in phosphate-buffered saline (PBS) containing antibiotics and assayed for the presence of HSV either by homogenisation or explantation; both carried out (with cocultivation on PRK monolayers) as described for sensory ganglia². Viral titres, expressed as the number of plaque-forming units (PFU) per ganglion, were determined on all homogenates positive for HSV.

The dilator muscle of the pupil is innervated by a rich network of adrenergic nerve terminals which have their cell bodies in the ipsilateral SCG¹¹. To expose these nerves to virus, 8×10^6 PFU of HSV suspended in 4 μ l were injected unilaterally into the anterior chamber of the eye using a 100- μ l syringe and repeating dispenser (Hamilton Co., Reno, Nevada); at various intervals thereafter the ipsilateral SCG was removed and assayed for virus. The data in Fig. 1*a* show that during the acute phase of infection, HSV was detected both in homogenates and explants of the SCG with maximum recovery of virus on days 5 and 6. The acute phase of infection ended at about day 8. Thereafter, homogenates were no longer positive, but HSV could still be recovered by explantation, indicating that the ganglia continued to harbour the virus.

To study the route of viral spread further, both ipsilateral and contralateral ganglia were assayed after unilateral inoculation of HSV into the anterior chamber of the eye. Comparison of bilateral homogenates (Fig. 1*b*) revealed that the amount of virus recovered from ganglia contralateral to the side of inoculation was less than 1% of that recovered from ipsilateral ganglia. Moreover, the occurrence of virus in explants of contralateral ganglia was delayed by 48–72 h compared with ipsilateral ganglia, in which virus could be detected as early as 24 h after inoculation (Fig. 1*c*). These studies indicate a direct, presumably neural, route of viral spread to the ipsilateral SCG. In contrast, spread to the contralateral SCG may involve an intermediary focus of infection (for example, pineal gland or brain parenchyma) although bilateral innervation of the iris or haematogenous viral spread cannot be ruled out.

Corneal inoculation with HSV (Table 1) led to a latent infection of the ipsilateral, but not contralateral, SCG (experiment A). The data from experiments B and C in Table 1 show that virus persisted in the autonomic ganglia for prolonged periods, lasting well over 1 yr. Since mice in experiments B and C were not tested earlier, the differences in the percentage of animals infected at 439 d compared with 114 d cannot be taken as indicating a decline in the prevalence of latent infection with time; assay of the trigeminal ganglia of animals in group C revealed a low prevalence of infection, suggesting a poor initial infection in this group.

The data in Table 2 show the effect of immunisation on the development of latent SCG infection. Mice were first immunised by intraperitoneal injection of HSV; 2 weeks later, when the mean serum neutralisation titre against HSV was 16, the animals were challenged with different doses of virus by anterior chamber inoculation. Mice were killed during the acute and chronic stages of infection, and the ipsilateral SCG was assayed for virus. High mortality and a latent infection of the SCG developed in unimmunised animals with a viral inoculum as low as 8×10^3 PFU. Immunisation prevented mortality and reduced by 80–100% the number of animals developing an infection of the SCG.

The events leading to HSV infection of the SCG can now be described. After anterior chamber inoculation, the virus probably contacts the abundant sympathetic fibres innervating the iris and passes by the neural route to the ipsilateral SCG. After corneal inoculation, either these same nerve fibres or those supplying the neighbouring blood vessels or lacrimal and nasopharyngeal glands serve as the point of viral entry leading to infection of the ipsilateral ganglion. The rapid development of HSV in the SCG supports the argument that virus can travel

epithelial surfaces innervated by the infected ganglia. The ganglia of the peripheral autonomic nervous system seem to share a common embryogenesis with sensory ganglia⁵. Although it has been shown that acute infection of autonomic ganglia with HSV⁶ and the related herpesvirus, pseudorabies virus^{7–9}, can occur, it has not been established that the peripheral autonomic nervous system (ANS) can support a latent infection with these or other viruses. We report here a murine model of latent infection of the superior cervical ganglion (SCG) of the sympathetic division of the ANS.

Four to six-week-old female BALB/c mice (Jackson Labora-

Table 1 Latent infection of the superior cervical ganglion with HSV after corneal inoculation

Experiment	Side of assayed ganglion	Days after inoculation	Assay of ganglion by explantation Superior cervical	Trigeminal
A	Ipsilateral	4	11/19 (58%)	9/9 (100%)
	Ipsilateral	26	6/17 (35%)	7/9 (78%)
	Contralateral	26	0/16 (0%)	ND
B	Ipsilateral	114	17/39 (44%)	10/10 (100%)
C	Ipsilateral	439	7/42 (17%)	4/18 (22%)

Mice were inoculated with HSV by corneal scarification, and at different times thereafter the SCG and trigeminal ganglia were assayed for virus by explantation. Results are expressed as the ratio of the number of ganglia positive for HSV to the number of ganglia tested. Mice in experiment A were inoculated unilaterally using a virus pool containing 2×10^8 PFU ml⁻¹. Mice in experiments B and C were inoculated bilaterally with virus pools containing 1×10^8 and 1×10^7 PFU ml⁻¹ respectively. In these latter experiments the results of both the right and left ganglia were combined; thus the number of animals tested in experiments B and C is equal to half of the number of ganglia recorded.

ND, Not done.

Table 2 Effect of immunisation on the acquisition of latent superior cervical ganglion infection with HSV after inoculation into the anterior chamber of the eye

Group	Virus challenge (PFU)	Deaths	Days after inoculation		
			6 (Homogenates)	6 (Explants)	21 (Explants)
Unimmunised	8×10^6	11/30	8/10	10/10	15/19
	8×10^5	18/30	6/10	10/10	10/12
	8×10^4	8/30	5/10	8/10	8/21
	8×10^3	14/30	3/10	8/10	6/15
Immunised	8×10^6	0/15	0/7	1/8	3/15
	8×10^5	0/15	1/8	1/8	0/15
	8×10^4	0/15	0/9	0/8	0/15
	8×10^3	0/15	0/8	0/8	1/15
	None			0/15	0/15

Mice were divided into two groups; one group received a single 0.1 ml intraperitoneal injection of live HSV containing 2×10^5 PFU ml⁻¹, while the other group was not immunised. Two weeks later the animals were further subdivided and challenged with different doses of HSV by the anterior chamber route. Ipsilateral ganglia of mice killed at 6 d after challenge were assayed both by homogenisation and explantation, while ganglia obtained 21 d after challenge, were assayed by explantation alone. Results are expressed as the ratio of the number of ganglia positive for HSV to the number of ganglia tested. Deaths occurred between 7 and 10 d after anterior chamber inoculation and were tabulated at 21 d; the mice killed at 6 d were randomly selected and omitted from computation of mortality.

by direct retrograde axonal transport¹². Once the virus reaches the ganglion, productive viral replication ensues, as indicated by rising viral titres in homogenates. Fluorescent antibody studies reveal that 5 d after inoculation more than half of the neurones in the SCG contain viral antigens (unpublished). At 8 d after inoculation, the SCG homogenates become negative and HSV antigen can no longer be detected by immunofluorescence. Nonetheless, the infection persists, and virus can be recovered by explantation for at least 14 months.

In spite of differences in the organisation and functional roles of the autonomic and sensory ganglia, it seems that both types can support a latent HSV infection. Whether either type of ganglion actually becomes infected probably depends on whether their respective nerve endings are exposed to an adequate concentration of virus. Likewise, whether immunisation can prevent infection may well depend on the availability of anti-viral antibody and/or immune cells at the point where virus makes contact with nerve endings¹³. Our study shows that immunisation is quite effective in reducing the incidence of SCG infection after anterior chamber inoculation.

HSV infection of the SCG promises to be a useful animal model for the study of latent viral infection of the ANS. Moreover, this study raises the possibility that latent infection of autonomic ganglia with HSV or other viruses may well take place in man and could be responsible for periodic or prolonged dysfunction of target organs innervated by these ganglia.

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Interval coding of temperature by CNS neurones in thermoregulation

MODELS describing the central nervous control of thermoregulation^{1,2} are based at present on the discharge characteristics of temperature rate-sensitive neurones in the preoptic (PO) area and the hypothalamus. These neurones are believed to be involved in thermoregulation because they are anatomically located in an area affecting thermoregulatory control³⁻⁵ and respond to both local⁶⁻⁸ and peripheral⁹⁻¹¹ temperature change. Examination of the temporal distribution of interspike intervals from PO neurones in our experiments suggests that temperature rate-insensitive PO neurones also process thermoregulatory information.

We used adult New Zealand White rabbits, *Oryctolagus*

cuniculus in our experiments. Several probes were stereotactically implanted¹² in the brain: a thermocouple re-entrant tube in the left lateral PO, a thermode¹³ and a thermocouple re-entrant tube in the lateral border of the right PO and a moveable recording electrode array¹⁴ in the right medial PO equidistant from the thermode and re-entrant tube. The recording electrodes were prepared from 25 μ m diameter platinum-iridium wire. Extracellular potentials were first conditioned by a field effect transistor¹⁵, mounted on the head of the animals, and then amplified and filtered through routine bioelectric equipment. Discharges were typically from single neurones. Neural activity, as well as ambient, ear, brain and PO temperatures were recorded on magnetic tape for subsequent analysis. Neural activity was analysed by a PDP 8-I laboratory computer and represented in the form of mean firing rate and interspike interval (ISI) histograms. The ISI histogram is a plot of the distribution of time intervals between successive neural discharges.

After surgery, the rabbits were allowed to recover for at least 1 week. During experimental sessions, they were placed in a controlled ambient temperature chamber and were not anaesthetised or restrained. After obtaining a unit with a suitable signal-to-noise ratio, control unit activity was recorded for comparison with data collected at hot or cool clamped PO and ambient temperatures.

The experimental ISI histograms were compared statistically with their respective control histogram using χ^2 analysis and the *F* test. In each case, the experimental values were significantly different ($P < 0.01$) from the control; also, histograms generated at normal PO temperatures after each episode of heating or cooling did not differ significantly from their control histogram ($P > 0.05$).

Figure 1 shows ISI histograms for data from rate-insensitive neurone 2-E7 at an ambient temperature of 22 °C. Although the firing rate did not change significantly ($Q_{10}=1.02$), there was a significant change in the distribution of intervals during PO cooling. Clamping the PO temperature at 35.8 °C resulted in a bimodal distribution of intervals.

The ISI histograms in Fig. 2 indicate that some rate-insensitive neurones respond to both local cooling and heating with a change in interval distribution. These data from rate-insensitive neurone 11-E2 were obtained at 24 °C ambient temperature. At normal PO temperature, the

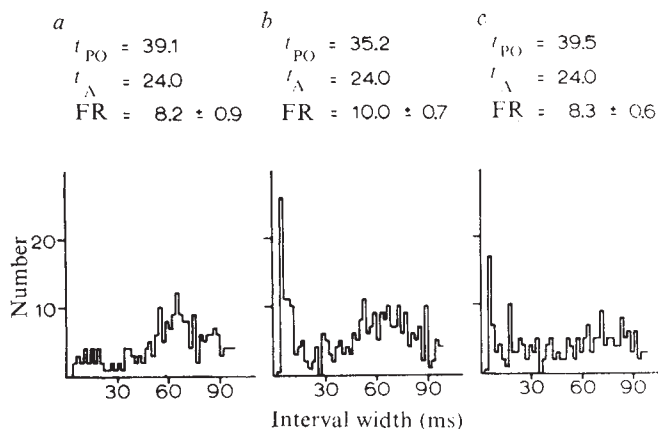


Fig. 2 Effects of preoptic cooling and heating on the interval distribution of rate-insensitive neurone 11-E2. *a*, Control; *b*, preoptic cooling; *c*, preoptic heating. Histogram details and definitions as in Fig. 1.

intervals were distributed unimodally, but during PO cooling or heating ($Q_{10}=0.6$ and 1.4 respectively), the intervals were distributed bimodally. In each case, the bimodal distribution resulted from the increased frequency of short intervals.

Since integrative thermoregulatory neurones receive information concerning external as well as internal temperature conditions, the ISI histograms were examined during ambient thermal stimulation. Figure 3 shows data from rate-insensitive neurone 17-L3 ($Q_{10}=0.9$). The interval distribution by this neurone is similarly by either PO or ambient thermal stimulation. The resulting bimodal distribution resembles that in Fig. 2 and was again a result of the increased frequency of short intervals. This type of neurone integrates thermal inputs from central and peripheral loci as expressed by its ISI distribution.

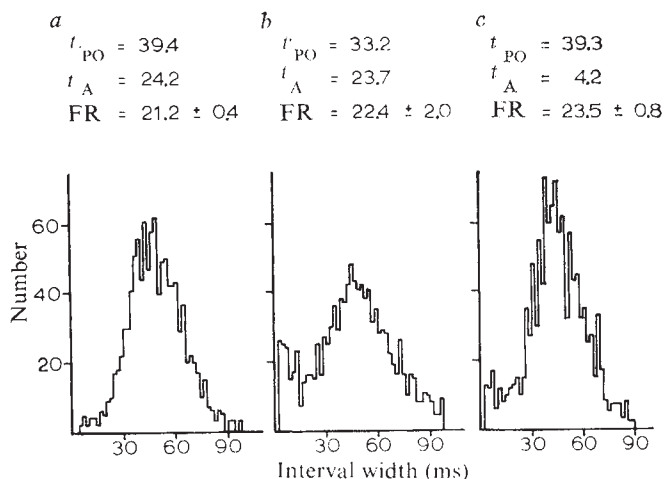


Fig. 3 Effects of ambient thermal stimulation on the interval distribution of rate-insensitive neurone 17-L3. *a*, Control; *b*, preoptic cooling; *c*, ambient cooling. Histogram details and definitions as in Fig. 1.

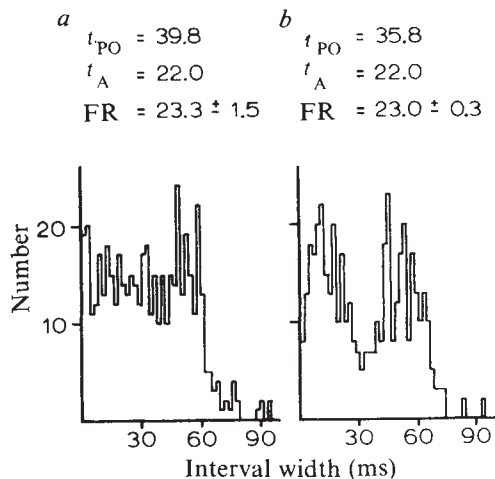


Fig. 1 Comparison of ISI histograms for rate-insensitive neurone 2-E7. *a*, Control; *b*, preoptic cooling. Each histogram represents 50 s of neural activity. The total number of occurrences of each interval width is expressed on the ordinate. Resolution of the interval width is 2 ms. t_{PO} , Preoptic temperature; t_A , ambient temperature; FR, firing rate (impulses s⁻¹ ± s.e.)

Models of the thermoregulatory system should include consideration of the importance of the ISI as a means of coding thermal information. In general the ISI histograms for these and other PO rate-insensitive neurones we have studied change significantly during thermal stimulation. Earlier studies^{16,17} indicated that only a small fraction (30%) of neurones in the PO are thermosensitive and considered to be involved with temperature regulation, however, firing rate was the only parameter used to determine

thermosensitivity and in addition, they dealt with anaesthetised animals. Consequently, our study may not conform to earlier results because our animals were capable of thermoregulation and not anaesthetised.

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Evoked transmitter release increased by inorganic mercury at frog neuromuscular junction

It is well documented that calcium ions have an important role in the release of transmitter substances from nerve terminals^{1,2}. There is, however, little information about the interactions of calcium with other chemical moieties within the nerve terminal which presumably are required to bring about transmitter release. Kosower and Werman³ postulated that calcium ions interact with two sulphhydryl groups to form a calcium dithiolate complex. The formation of such a complex results in a decrease in the sulphur-sulphur distance which is presumed to produce a micro-contraction of the presynaptic membrane, thereby allowing for the quantal release of transmitter into the synaptic cleft. We show here that low concentrations of mercury ions (1 μ M or less) increase the amount of transmitter released from motor nerve terminals. Since it is well known that mercury ions dissociating from inorganic mercury (HgCl_2) form disulphide bridges in the presence of sulphhydryl groups, we interpret our observations as being consistent with Kosower and Werman's hypothesis.

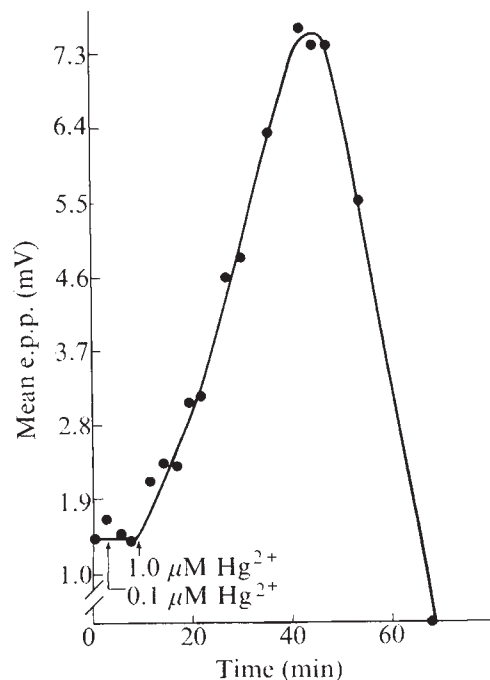
Experiments were performed *in vitro* on frog (*Rana pipiens*) sciatic nerve-sartorius muscle preparations maintained in a suitable chamber. Superficial neuromuscular junctions were viewed through a compound microscope with a total magnification of $\times 400$. Subthreshold endplate potentials (e.p.p.s) were produced by bathing the preparations in high magnesium/low calcium Ringer solution. The composition of the control Ringer solution was: 111 mM NaCl, 2.5 mM KCl, 0.36 mM CaCl_2 , 1.1 mM MgCl_2 , 4 mM Tris-maleate. Micromolar amounts of HgCl_2 were added to this Ringer solution to test for both presynaptic and postsynaptic effects of mercury ions. All Ringer solutions were maintained at pH 6.9 and 15 °C. Microelectrodes filled with 1 M potassium citrate were used for intracellular voltage measurements. A signal averager (Computer of Average Transients 1000) was used to record the mean e.p.p. To determine whether changes in the e.p.p. were

due to a direct effect of mercury ions on the postsynaptic receptors, these receptors were activated directly through the iontophoresis of acetylcholine (ACh) from a micropipette rather than indirectly by nerve stimulation, which results in the neural release of ACh.

Figure 1 shows that mercury had an intense potentiating effect on the e.p.p. amplitude. In this experiment, the mean e.p.p. amplitude was recorded during prolonged exposure of the preparation to 1.0 μ M Hg^{2+} . The e.p.p. increased from a control level of 1.4 mV to a value near 7.3 mV within 30–40 min of the beginning of the mercury exposure. The addition of 0.1 μ M Hg^{2+} a few minutes before the addition of the 1.0 μ M Hg^{2+} resulted in no detectable change in the e.p.p. amplitude. The percentage increase from control after addition of 1.0 μ M Hg^{2+} was 420%. The e.p.p. amplitude did not remain steady at this value of 7.3 mV, but it soon began to fall, eventually to zero. The increase in e.p.p. amplitude brought about by Hg^{2+} is very similar to the effect of diamide on the e.p.p. observed by Werman *et al.*⁴. These investigators suggest that diamide increases the e.p.p. amplitude by the formation of disulphide bonds in the presynaptic nerve terminal, which, according to their hypothesis, results in an increased quantal release of transmitter.

The potentiating effect of mercury ions on the e.p.p. occurs when mercury is present in low concentrations—either 1.0 μ M or less. In one experiment, 0.01 μ M mercury increased the e.p.p. by 33%; in another, 0.1 μ M mercury increased the e.p.p. by 26%. In the latter experiment, washing the preparation with mercury-free Ringer solution resulted in only a slight fall of the e.p.p. towards the control

Fig. 1 Effect of the e.p.p. of a prolonged exposure to 1.0 μ M HgCl_2 . The amplitude of the mean e.p.p. is plotted on the ordinate against time. Each point represents the mean of 100 e.p.p.s. E.p.p.s were recorded by conventional intracellular microelectrode techniques, and neuromuscular blockade was produced by bathing the preparation in high magnesium/low calcium Ringer solution. During the averaging of the e.p.p., the motor nerve was stimulated at a rate of two per s. 0.1 μ M HgCl_2 caused no detectable change in the e.p.p. amplitude; however, the addition of 1.0 μ M HgCl_2 increased the e.p.p. amplitude by 420% from the control value. The e.p.p. amplitude did not remain constant during the prolonged exposure to 1.0 μ M mercury ions; after reaching its peak amplitude, the e.p.p. amplitude eventually fell to zero. The concentration of Ca^{2+} was 0.36 mM and of Mg^{2+} , 1.1 mM. The temperature was 15 °C.



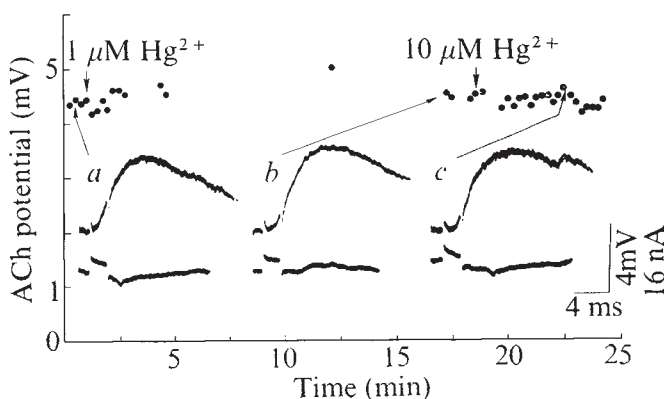


Fig. 2 Lack of a postsynaptic effect of HgCl_2 . ACh was iontophoretically applied to the endplate receptors. The amplitude of the ACh potential was plotted on the ordinate against time. The oscillogram in *a* shows the control ACh potential (upper trace) and the corresponding iontophoretic current in the lower trace. Records in *b* and *c* show that, respectively, 1.0 and 10.0 μM HgCl_2 had no obvious effect on the amplitude of the ACh potential. Each point on the plot represents the amplitude of a single ACh potential. Note the presence of a single m.e.p.p. on the falling phase of the ACh potential in *c*; 10.0 μM HgCl_2 , as in other experiments, increased the m.e.p.p. frequency. The concentration of Ca^{2+} was 0.36 mM and of Mg^{2+} , 1.1 mM. The temperature was 15 °C.

value, illustrating our consistent finding that this effect of mercury is irreversible. When this same preparation was exposed to 10.0 μM mercury, the e.p.p. at first increased further, then decreased quickly to zero.

del Castillo *et al.*⁵⁻⁷ have shown that several types of sulphhydryl reagents including oxidising agents, organic mercurials and divalent mercury ions depolarise the endplate membrane. They suggest that activation of the endplate involves a change in certain membrane sulphhydryl groups from their initial reduced form, a process essentially identical to that proposed for presynaptic transmitter release by Kosower and Werman (see ref. 3). To determine whether the effects of HgCl_2 on the e.p.p. amplitude in our experiments were presynaptic or postsynaptic in origin, the amplitude of the iontophoretic potential (ACh potential) produced by a constant amount of ACh released from a micropipette was observed before and during exposure of the preparation to HgCl_2 . Identical Ringer solutions were used during the control periods of the experiments illustrated in Figs 1 and 2.

Figure 2 contains a plot of the amplitude of the ACh potential (ordinate) against time; measurements of the amplitude were obtained before and after the application of 1.0 μM and 10.0 μM mercury ions. Figure 2*a*, *b* and *c* are representative oscillograms of these ACh potentials. The ACh pipette was highly localised at the intrinsic receptors⁸; the ACh sensitivity⁹ was quite high, the value being 787 mV nC⁻¹ for the recording made during the control period (Fig. 2*a*). Mercury caused no great increase in the amplitude of the ACh potential; the ACh potential measured in 10.0 μM mercury (Fig. 2*c*) was 4.2% higher than that measured during the control period (Fig. 2*a*). Clearly then, the massive increase in the e.p.p. amplitude observed in the experiment of Fig. 1 was due to a presynaptic effect of mercury ions.

We conclude that 1.0 μM mercury increased the amount of transmitter released from the nerve terminal after the arrival of an action potential, and that the large increase in the e.p.p. amplitude was due principally to this potentiating effect of the low concentration of mercury on evoked transmitter release. These experiments suggest that sulphhydryl groups are involved in the release of transmitter from nerve terminals. We suggest that, at very low con-

centrations of HgCl_2 , mercury dithiolate bridges form within the presynaptic nerve terminal as the presynaptic action potential arrives. In these conditions, mercury ions are presumed to act like calcium ions with regard to the evoked release of transmitter. But as the concentration of mercury ions is increased or the exposure time to a relatively low concentration of mercury ions is increased, transmitter release is blocked, presumably because the bonds between sulphur and mercury are relatively irreversible and do not allow for thiol groups to be reformed as they would in the presence of calcium ions.

The frequency of miniature endplate potentials (m.e.p.p.s) was unaffected by mercury concentrations of 1.0 μM or lower. Only at concentrations of 10 μM or more—concentrations at which the e.p.p. was usually blocked completely—did we observe an increase in m.e.p.p. frequency. Methyl mercury chloride has also been observed to produce an increase in m.e.p.p. frequency as well as neuromuscular blockade¹⁰. Thus, the action of mercury ions on the evoked release of transmitter occurs at much lower concentrations than its effects on spontaneous release. This is similar to the effects of PbCl_2 on the neuromuscular junction¹¹ except that low concentrations of PbCl_2 only decreased the e.p.p., never increased it.

It is uncertain what direct bearing the results of these experiments may have on neuromuscular disorders associated with chronic mercury poisoning. It is interesting, however, that a study¹² of methyl mercury poisoning showed that patients benefit from the administration of neostigmine, an anticholinesterase agent. Thus, although other interpretations are possible, it may be that disturbances in the release of acetylcholine from motor nerve terminals may prevail in chronic mercury poisoning.

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Effect of internal fluoride and phosphate on membrane currents during intracellular dialysis of nerve cells

It has become possible recently to isolate single neurones from molluscan ganglia by the use of proteolytic enzymes, so that the external solution can be changed easily^{1,2}. One advantage is that the absence of the axon makes the application of the voltage clamp technique to isolated neurones more effective³. We describe here a further development—an attempt to produce wide ranging changes in the ionic com-

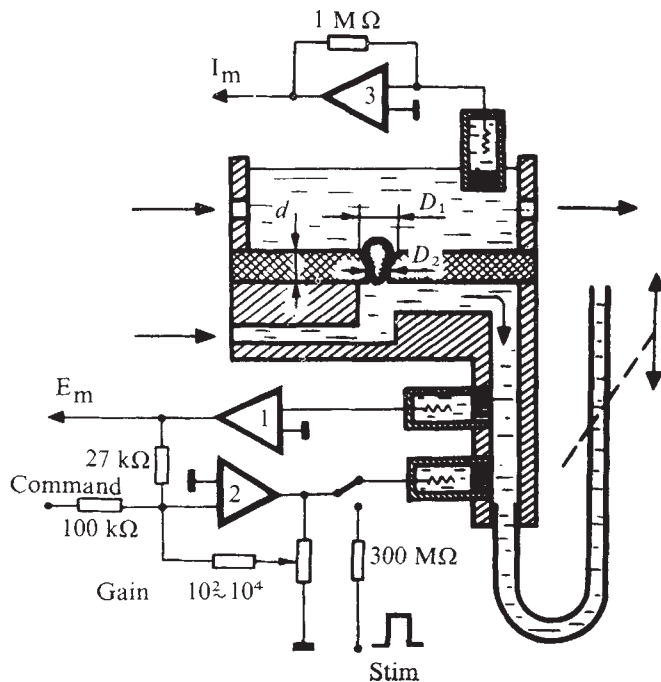


Fig. 1 Experimental design for intracellular dialysis and voltage clamping. The dimensions of the pore were: $D_1 = 200 \mu\text{m}$, $D_2 = 40 \mu\text{m}$. Polyethylene film has a thickness d of $400 \mu\text{m}$. The suction in the pore is regulated by changing the level of the outflow as shown by a vertical arrow. Electric circuits: 1, unity gain input amplifier; 2, amplifier for voltage clamping; 3, current-measuring amplifier.

position of the intracellular medium of neurones isolated from the snail *Helix pomatia*.

The experimental design is shown in Fig. 1. The perfusion chamber consists of two compartments separated by a polyethylene film with a conical pore. If a small negative hydrostatic pressure is created in the lower compartment, a suction effect is created in the pore. When an isolated cell is transferred to the solution in the upper chamber, it plugs the pore and becomes fixed in it. The walls of the pore are covered with an adhesive material made from Parafilm which helps to reduce leakage between the cell membrane and the walls of the pore. The part of the cell membrane that is exposed to the lower

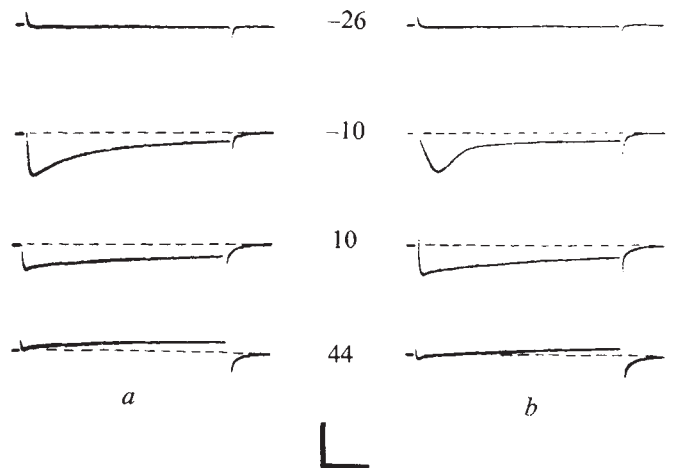
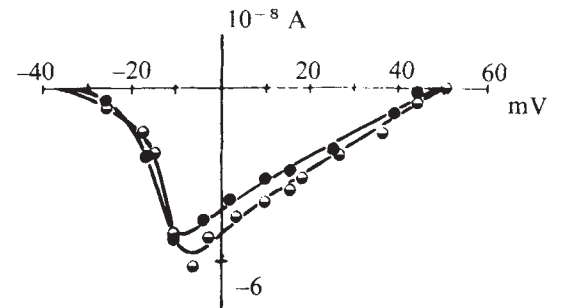


Fig. 3 Currents and $I-E$ relationship for the peaks of inward current in the cell containing phosphate anions. Solution in the lower compartment: 100 mM $\text{Tris-H}_3\text{PO}_4$, pH 7.3, 20 mM sucrose. Upper compartment: normal Ringer (a, $\circ$ on the $I-E$ curve); Na-free solution (b, half-closed circles). Leakage was compensated for in this experiment as described in the text. Holding potential was -40 mV . Calibrations: $6 \times 10^{-8} \text{ A}$, 100 ms.

compartment is subjected to the action of calcium-free iso-osmotic (or slightly hypo-osmotic) potassium salt solution, pH 7.3, which is perfused through this compartment; this rapidly destroys the barrier properties of the membrane. (The presence of potassium is not critical in this respect since the absence of calcium itself is usually sufficient.) The upper part of the surface membrane which is in contact with normal Ringer solution (100 mM NaCl , 10 mM CaCl_2 , 2 mM KCl , 10 mM Tris-HCl , pH 7.5) in the upper compartment usually maintains its electrical excitability.

Being fixed in a pore, the cell membrane is electrically controlled with the help of the $\text{Ag/AgCl/saturated KCl}$ electrodes connecting the solutions in both compartments to the voltage clamp device as shown in Fig. 1. Resultant membrane potential values of -20 to -30 mV can be recorded between

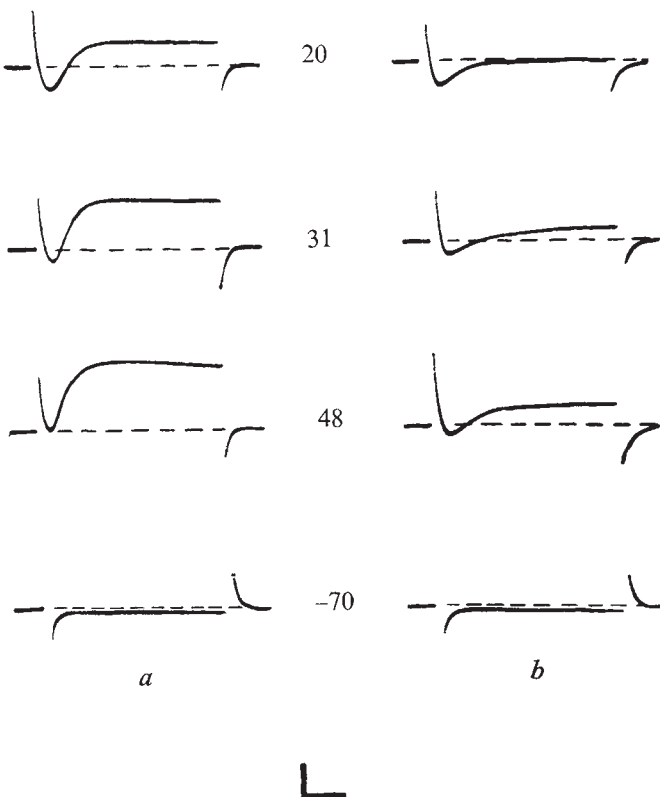


Fig. 2 Effect of a decrease in the internal potassium concentration. Potassium concentration in the lower compartment was decreased from 60 mM (a) to 2 mM (b). Osmolarity was maintained with Tris-HF (pH 7.3). Normal Ringer solution was in the upper compartment. Membrane potentials at test pulses (in mV) are indicated by numbers near the corresponding current traces. Holding potential was -40 mV . Calibrations: $4 \times 10^{-8} \text{ A}$, 10 ms.

the two compartments showing that leakage is not more than twice as great as that normally observed in the isolated cells.

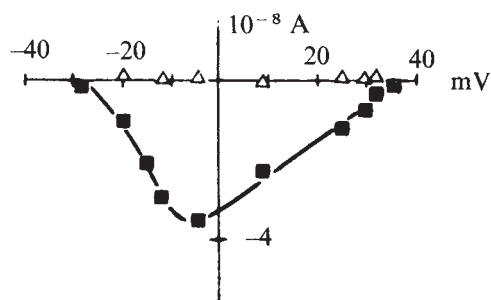
On repolarisation to a normal resting potential (-40 to -50 mV), the upper part of the membrane produces either reduced or, in many cases, full sized action potentials.

Thus we were able to dialyse the cell body and so change the intracellular ionic composition. The effectiveness of dialysis was tested first by replacing the potassium in the lower compartment by Tris. This substitution resulted in a quick (1–5 min) and marked decrease in the delayed outward current carried by potassium ions (Fig. 2).

The stability of the membrane depended largely on the kind of anions in the dialysing solution. In 100 mM KCl (or Tris-HCl) the membrane could not maintain its initial condition for sufficient time to facilitate its study. The leakage current grew continuously and the ionic currents deteriorated. NO_3^- was even less favourable in this respect. With fluoride or phosphate salts, however, the membrane showed a remarkable stability which exceeded that found during usual micro-electrode experiments on intact cells. The leakage conductance was stable for hours and did not depend on potential within the range of potential changes used. Thus we could compensate for leakage by adding to the current signal a square pulse proportional to the potential step.

The properties of the inward current were strikingly different

Fig. 4 Currents and $I-E$ relationship for the peak inward current in the cell containing fluoride anions. Lower compartment: 100 mM Tris-HF, pH 7.3, 20 mM sucrose. Upper compartment: normal Ringer (a, $\blacksquare$); Na-free solution (b, $\triangle$). Leakage was compensated for. Holding potential was -40 mV. Calibrations: 3×10^{-8} A; 20 ms.



depending on whether fluoride or phosphate anions were introduced into the cell. When dialysed with phosphate salts the cells developed inward currents which were independent of external sodium and carried only by calcium ions. When external sodium was replaced by Tris there was no decrease in the inward current, but a slight increase could be observed, as if there was a degree of potential-dependent blocking of inward current channels by sodium (Fig. 3). In cells containing fluoride, however, the inward membrane current was carried entirely by sodium, disappearing after replacement of this ion (Fig. 4). An increase in external calcium did not abolish this effect. A cell dialysed first in the presence of phosphate salts and demonstrating calcium inward currents could be made to transport sodium by the introduction of fluoride. In neither state were the inward currents sensitive to tetrodotoxin, and the slope conductances of their steady state $I-E$ relationships were similar, indicating that probably the same system of channels is involved in the transfer of either calcium or sodium ions. The change of the inward current channels was not completely reversible: the change back from fluoride to phosphate was followed by a sharp decrease in maximum current.

The calcium current detected in the cells containing phosphate had kinetics different from those of the sodium current in the cells containing fluoride. The latter inactivated completely, the time course being exponential (Fig. 4), whereas the former had a small, very slowly inactivating component (Fig. 3). This component may be similar to a slow calcium current recently observed in unisolated neurones⁴.

The reversal potential for this late component was 20–30 mV less positive than for the early inward current which reversed at about +50 mV (Fig. 3). This could be due to its contamination by a residual small potassium current which could be observed at high depolarisation.

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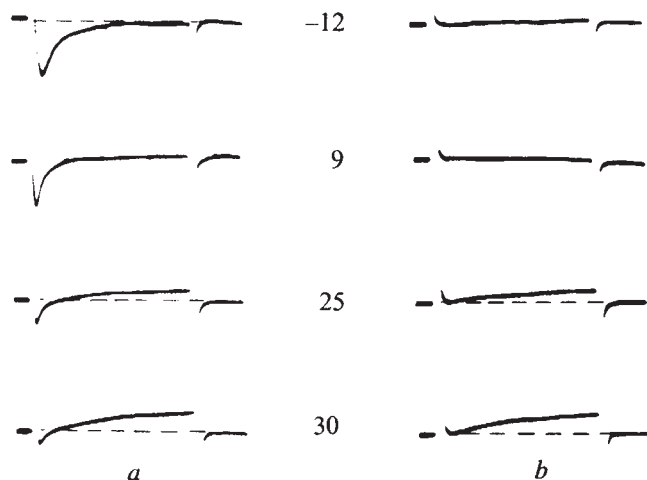
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Simultaneous changes in fluorescence and optical retardation in single muscle fibres during activity

WHEN a skeletal muscle is stimulated to twitch, small changes in the optical properties of the fibres can be detected which may give information about excitation-contraction coupling. An example is provided by the experiments of Bezanilla and Horowicz^{1,2}. After treatment of muscles with Nile Blue A, fluorescence changes were found which, it was suggested, arise from changes in potential across the membranes of the sarcoplasmic reticulum (SR). Such potential changes might reflect the movement of calcium ions from inside the SR into the myoplasm, a step in the normal activation of contraction. Baylor and Oetliker^{3,4} measured changes in optical retardation, which rely on the inherent birefringence of a muscle fibre rather than the addition of an extrinsic probe. The large second component of the signal was suggested to arise from a change in SR membrane potential. These explanations are plausible since small changes in both extrinsic fluorescence



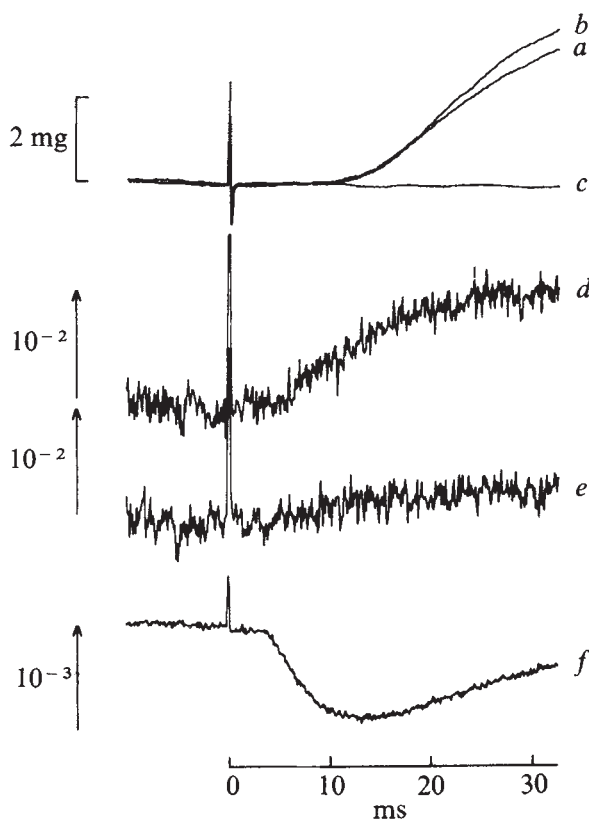


Fig. 1 Tension, Nile Blue A fluorescence and optical retardation in a single muscle fibre during a twitch. Records *a–c* show twitch tension recorded in Ringer (*a*), 18 min after introducing Ringer + Nile Blue A ($0.0002 \text{ mg ml}^{-1}$) (*b*), and 10 min after replacing with 270 mM NaCl Ringer + dye ($0.0002 \text{ mg ml}^{-1}$) to block movement (*c*). The tension recorded in isotonic solution was relatively low since the fibre was small, diameter $56 \mu\text{m}$, and was stretched to 1.55 times slack length. Records *d–f* show optical measurements made after the hypertonic Ringer was introduced. *d*, Change in fluorescence expressed as change in light intensity divided by resting fluorescent light intensity, using light of $570 \pm 30 \text{ nm}$ to illuminate the fibre (determined by primary filter) and measuring light of wavelengths longer than 630 nm (determined by secondary filter). *e*, Fluorescence control expressed as change in light intensity divided by the resting intensity used in *d*, obtained by placing the secondary filter between the primary filter and the fibre. *f*, Change in optical retardation using light of $570 \pm 30 \text{ nm}$. For records *a*, *b* and *f*, 16 traces were signal averaged; for *c*, *d* and *e*, 32 traces were averaged. In each record the fibre was stimulated 10 ms after the beginning of the sweep as indicated by the arrows. An upward deflection in the optical traces represents an increase in light intensity, as indicated by the arrows. Temperature, $12\text{--}13^\circ\text{C}$.

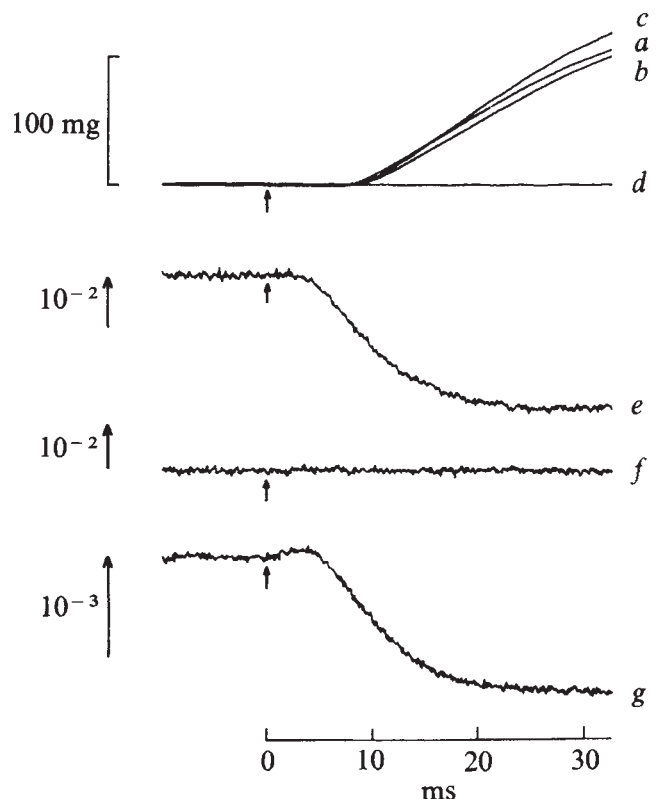
and optical retardation are known to accompany voltage changes across neuronal membranes^{5–8}. An important test of these hypotheses is to compare the fluorescence and retardation signals from the same fibre, to see if they follow the same time course.

Single fibres from the semitendinosus or ilio-fibularis muscle of English frogs (*Rana temporaria*) were isolated and mounted horizontally in a narrow chamber with glass sides. One tendon end was connected to an RCA 5734 transducer for monitoring tension. The chamber was then placed on an optical bench (Spindler-Hoyer) where the light from a tungsten-halogen source could be focused on the fibre by a long working distance objective ($\times 32$, N.A. 0.60). An identical objective was placed on the opposite side to collect the transmitted and emitted light. Light collected from 0.3 mm of fibre was measured by a photodiode (PV-100, E.G. & G., Inc.). The tension transducer and the photodiode were connected to Tektronix 3A9 amplifiers, bandwidth d.c. to 3 kHz, and the outputs were sampled and

signal averaged on-line by a PDP-8/e computer. Measurements of fluorescence were made using a wide-band interference filter (primary filter) placed between the light source and the muscle fibre and a longer wavelength cut-on filter (secondary filter, Schott R.G. series) placed between the fibre and the photodiode. Optical retardation was measured with two polarisers placed on either side of the fibre, one at -45° and the other at $+45^\circ$ with respect to the fibre axis⁴. A shutter limited the exposure of the fibre to light. For data collection the fibre was stimulated externally with 0.2 ms pulses at a rate of $0.1\text{--}0.2 \text{ s}^{-1}$. Only fibres that gave all-or-nothing mechanical and optical responses were used. Optical recording was usually done about 1 mm from the cathod. The Ringer solution contained 120 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2 , 2.15 mM Na_2HPO_4 and 0.85 mM NaH_2PO_4 , pH 7.1 (ref. 9). Mechanical movement was sometimes blocked by increasing the NaCl concentration to 270 mM, thus making the tonicity of the solution about 2.2 times normal.

In the first experiments with Nile Blue A it became apparent that single fibres do not survive well in the concentration used successfully on whole muscle^{1,2}, 0.02 mg ml^{-1} . In one experiment a 20-fold dilution produced a potentiation of the twitch of about 50% in 10 min, which was

Fig. 2 Tension, indodicarbocyanine fluorescence and optical retardation during a twitch. Records *a–d* show twitch tension in Ringer (*a*), 14 min after introducing Ringer + 0.002 mg ml^{-1} of the dye (*b*), 13 min after returning to normal Ringer (*c*), and 5 min after replacing with 270 mM NaCl Ringer (*d*). The fibre was in the dye solution for 19 min, then in isotonic Ringer for 14 min before hypertonic Ringer was introduced. Records *e–g* show singlesweeps of changes in fluorescence (*e*), fluorescence control (*f*), and changes in optical retardation (*g*), all made after hypertonic Ringer was added. For fluorescence measurements the primary filter passed light of $570 \pm 30 \text{ nm}$ and the secondary filter passed light of wavelengths longer than 665 nm . Optical retardation was measured using light of $570 \pm 30 \text{ nm}$. Stimulation time indicated by arrows. Fibre diameter $151 \mu\text{m}$; stretched 1.32 times slack length. Temperature $14\text{--}16^\circ\text{C}$. The later portion of the optical retardation signal in this experiment is different from that shown in Fig. 1, the reason probably being related to degree of stretch (unpublished results of S.M.B. and H.O.).



reversed within 15 min after removing the dye. Since a 100-fold dilution, $0.0002 \text{ mg ml}^{-1}$, had little or no potentiating effect, this concentration was used for optical recording. Record *a* in Fig. 1 shows tension recorded in Ringer solution, record *b* in Ringer plus dye, and record *c* after 270 mM NaCl Ringer plus dye was introduced to minimise movement artefacts on the optical traces. Signal-averaged records of fluorescence (*d*) and of optical retardation (*f*) were each taken with the fibre in hypertonic solution. The initial phases of both signals are qualitatively similar to the signals observed at normal tonicity, except that hypertonicity reduced the maximum rate of change by a factor of 4 for optical retardation⁴ and about 3 for fluorescence. The change in light intensity in record *d* represents primarily a change in fluorescent light from Nile Blue A since the signal was not seen without the dye and was greatly reduced when the secondary filter was placed between the primary filter and the muscle fibre, record *e* ('control' trace). Similarly, the signal in record *f*, corresponding to Baylor and Oetliker's second component⁴, represents a change in optical retardation since it can be reversed by adding an appropriate amount of compensation (not shown). The conclusion from the experiment is that the fluorescence and optical retardation signals are similar in appearance, at least initially, but that the noise level of the fluorescence traces *d* and *e* is too high to permit accurate quantitative comparison. For this reason, it was desirable to find a fluorescent dye that would give a larger signal, without otherwise affecting the fibre.

A marked improvement in the signal-to-noise ratio was obtained with an indodicarbocyanine dye (dye 122 in ref. 7) which could be used in concentrations up to 0.002 mg ml^{-1} with little effect on the twitch. Record *a* in Fig. 2 shows tension recorded in Ringer; *b*, after introducing the dye; *c*, after returning to Ringer; *d*, after replacing with 270 mM NaCl Ringer. Record *e* shows a single fluorescence trace (that is, not signal averaged), *f* shows a single fluorescence control trace and *g* shows a single optical retardation trace, all taken with the fibre in hypertonic solution. Apart from the slight hump in trace *g*, which occurs just before the downward deflection, traces *e* and *g* are remarkably similar. The cause of the hump, which was seen only when dye was used, is not understood. In this experiment the change from isotonic to hypertonic solution reduced the maximum rate of change of both optical signals by a factor of 6.

Since a merocyanine dye (dye I of ref. 7, available as Merocyanine 540, Eastman Organic Chemicals) has been reported to give a fluorescence signal originating mainly from T-system membrane¹⁰, it was interesting to try this dye as well. Figure 3 shows results from two experiments. *A* shows signal-averaged records obtained in isotonic Ringer plus dye. Trace *a* shows tension, *b* shows fluorescence, and *c* gives the control for fluorescence. Although movement artefacts became apparent once tension started to develop, it is clear that the fluorescence signal was early and transient, unlike the fluorescence signals obtained using the other dyes. In other experiments, the merocyanine signal propagated with a velocity comparable with that expected for an action potential, making it unlikely that it represents some sort of movement artefact. This merocyanine dye is toxic⁷ to single muscle fibres, however. Similar experiments in hypertonic solution were even more difficult to carry out. Figure 3*B* shows our best attempt. Record *a* indicates tension, *b* and *c* show fluorescence and fluorescence control, respectively, and *d* shows optical retardation. It is apparent that the change in fluorescence precedes the large change in optical retardation, a finding consistent with the idea that merocyanine is an indicator of surface and T-system membrane potential.

The general conclusion from these experiments is that the second component of the optical retardation signal and the fluorescence signal from Nile Blue A or an indodicarbo-

cyanine dye have approximately the same time course, making it likely that they are indicators of the same basic process or processes. Since changes in membrane potential can give rise to both types of optical signals, the similarity of the signals might be considered as evidence for such a mechanism in muscle. If so, the arguments of Bezanilla and Horowicz² and Baylor and Oetliker⁴ make the SR membrane the likely candidate. Although this view is attractive,

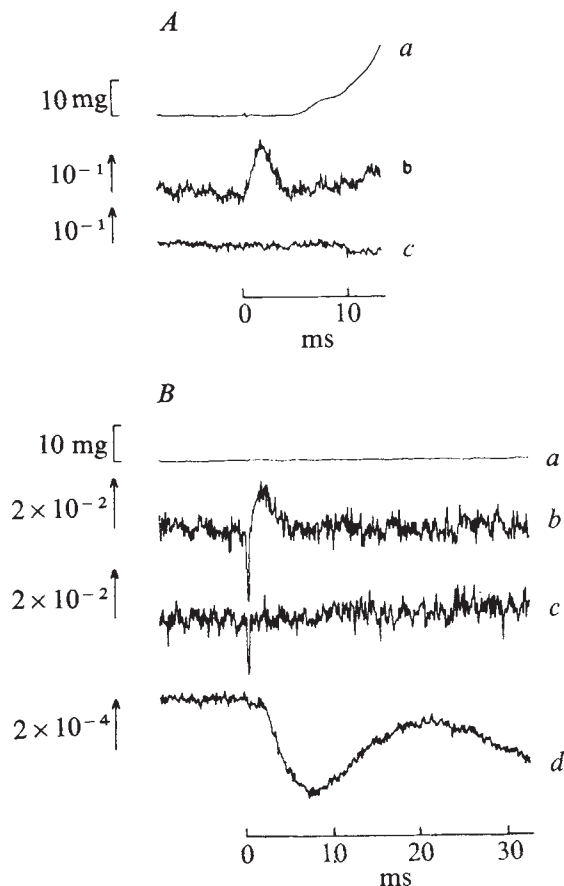


Fig. 3 Tension, fluorescence and optical retardation using a merocyanine dye. *A*, records made in isotonic Ringer saturated with the dye. Trace *a* shows tension, *b* shows change in fluorescence and *c* shows the fluorescence control; four sweeps were signal averaged for *a* and *b*, 16 for *c*. The traces were cut off at the time when large movement artefacts started to develop on the optical traces. Fibre diameter and stretch were not recorded. *B*, records obtained from a different fibre in 270 mM NaCl hypertonic Ringer without dye following 30 min in normal Ringer + dye. The dye in the soaking solution was dissolved in ethanol and Pluronic F-127 (BASF Wyandotte, as described in ref. 7) before being added to Ringer to give a final concentration of 0.018 mg per ml of dye, 0.2% ethanol and 0.02% Pluronic. Just before use the solution was filtered through a 0.22- μm Millipore filter. Trace *a* shows tension, *b* shows fluorescence change, *c* gives the fluorescence control and *d* gives the change in optical retardation using wavelengths longer than 665 nm; eight sweeps were signal averaged for *a*, *b*, *c* and four sweeps were averaged for *d*. Fibre diameter 123 μm ; stretched 1.31 times slack length. For the fluorescence measurements in *A* and *B* the primary filter passed light of $570 \pm 30 \text{ nm}$ and the secondary filter passed wavelengths longer than 630 nm. All solutions were bubbled with N_2 . These experiments were carried out at room temperature, 19°C in *A* and 21°C in *B*.

other explanations should also be considered. For example, optical signals might accompany an increase in myoplasmic calcium or a possible change in SR volume related to calcium release. In any event it is encouraging that fluorescence as well as birefringence methods can be made to work on single muscle fibres without the use of signal

averaging, and that different dyes might be used to follow different steps in muscle activation.

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Membrane noise in slowly adapting stretch receptor neurone of lobster

ANALYSIS of membrane noise in excitable membranes may yield information about the kinetics governing the conductance control of various ionic channels. Such analysis has been applied to noise arising from conductance fluctuations in, for example, the potassium system^{1,2} or the system of postsynaptic ionophores^{3,4}. This report deals with noise that seems to be generated in the sodium channel of the slowly adapting stretch receptor neurone of lobster. We recorded this noise as current noise under voltage clamp, and subsequently analysed it with respect to its covariance and spectral density function. A particularly low degree of Na inactivation at polarisations down to firing threshold was inferred from these analyses.

We used isolated cells mounted in a chamber continuously perfused with solutions of desired composition at 18 °C. For voltage clamp, the same low resistance (5–8 M Ω) microelectrode, filled with 3 M KCl, was used for measuring the membrane potential and passing the potential controlling currents. This we did by subtracting any potential artefact due to current flow in the electrode from the output signal of the input amplifier before passing it on to the control amplifier. Tests with electronic circuits simulating the membrane and microelectrode showed that a satisfactory signal subtraction of this kind could be achieved for control currents up to 5 nA in a frequency range of 0–250 Hz. After bandpass filtration (cut-off frequencies at 1.6 and 350 Hz), the recorded current noise was processed on a PDP-15 computer for determination of the covariance function and its Fourier transform (spectral density function) between 2 and 400 Hz.

A distinct voltage noise was seen in the slowly adapting stretch receptor neurone of lobster at subthreshold conditions. This noise increased with increasing depolarisation and became indiscernible only after polarisation beyond resting potential. Under voltage clamp the corresponding current noise exhibited a similar polarisation dependence (Fig. 1a and b). The membrane noise was abolished by addition of 120 nM tetrodotoxin or by complete substitution of choline or Tris for extracellular Na⁺ (Fig. 1c and d). The noise was unaffected, however, by 20 mM tetraethyl-

ammonium (Fig. 1e and f), whose ability to block the K channel was manifested by elimination of the after-hyperpolarisation of the action potential. These pharmacological tests show that the Na system was involved in the generation of noise in our preparation.

For a more detailed analysis of the noise generating process, covariance and spectral density functions were computed for current noise recorded at different levels of membrane polarisation. The results of such analyses showed that for more moderate depolarisations the computed covariance and spectral density functions agreed well with theoretical values typical of a first-order noise generating process (Figs 2 and 3). For depolarisation close to firing threshold, however, the computed covariance function deviated in a sinusoidal fashion from an exponential development (Fig. 2), and the spectral density function formed a hump superposed on the simple $[1 + (f^2/f_0^2)]^{-1}$ relationship (Fig. 3). It also seemed that with depolarisation there was an increase in time constant of the covariance function and a decrease in half-power frequency (Fig. 3) of the spectral density function. Finally, the noise variance (that is the covariance function at zero time) increased at an increasing rate with depolarisation.

The more complicated behaviour of the computed covariance and spectral density functions in near-threshold conditions was interpreted to indicate the presence of some periodic signals in the noise. In our preparation such signals could arise in connection with a subthreshold repetitive activity in peripheral cell regions where voltage was not well controlled and will not be considered here.

With regard to the first-order process which seems to underlie noise generation at all lower, but presumably also at higher depolarisations, it is assumed to be identical with the gating process of the Na channel. This gating process can be envisaged as some kind of state transition in channel gating elements, whereby the transition intensity obeys first-order kinetics^{5,6}. Measurements of so-called gating currents have verified the presence of first-order kinetics, at least as far as state transitions in the Na activating system are concerned^{7,8}.

For a more detailed interpretation of our noise recordings we presume that the Na channel is gated by an activating (*m*) and by an inactivating (*h*) system⁹. Provided that both of these systems are subject to random state variations, which is likely from thermodynamic principles, both will generate noise components (henceforth called *m* and *h*-noise

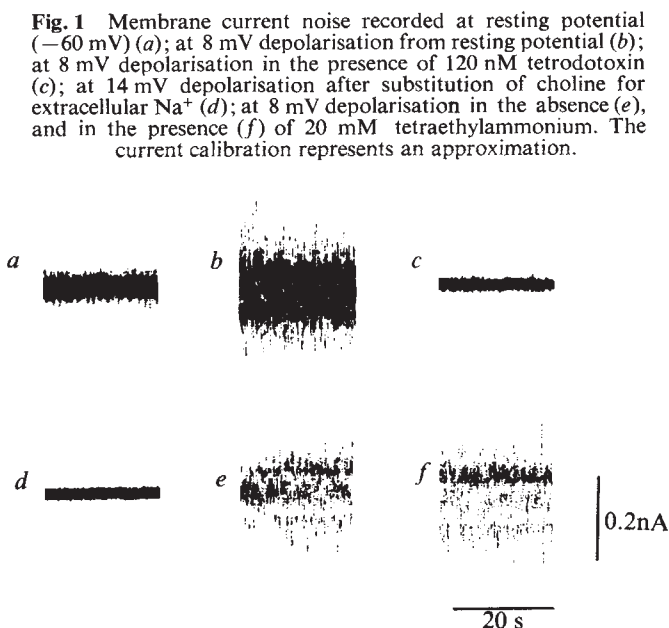


Fig. 1 Membrane current noise recorded at resting potential (–60 mV) (a); at 8 mV depolarisation from resting potential (b); at 8 mV depolarisation in the presence of 120 nM tetrodotoxin (c); at 14 mV depolarisation after substitution of choline for extracellular Na⁺ (d); at 8 mV depolarisation in the absence (e), and in the presence (f) of 20 mM tetraethylammonium. The current calibration represents an approximation.

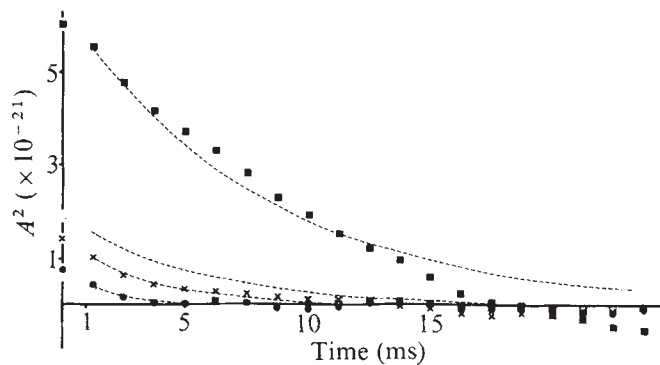


Fig. 2 Covariance function (ordinate) computed for current noise recorded at resting potential (-70 mV) ($\bullet$) and at 12 mV ($\times$), 13 mV ($\square$), and at 14 mV ($\blacksquare$) depolarisation from resting potential, and at 16 mV depolarisation in the presence of 120 nM tetrodotoxin ($\circ$). ---, Theoretical values which are proportional to $\exp(-|\Delta t|/\tau)$, where Δt represents the time lag (abscissa) and τ a time constant whose value varies between 1.6 and 7.9 ms.

components) which then combine in the formation of the membrane noise.

If it is assumed that, in subthreshold conditions, the time constants for state transitions in the m and h system are of the order of 0.1 – 0.3 ms, and 1 – 10 ms, respectively⁹, it follows that the bandwidth of the m noise will extend well beyond 500 – $1,500$ Hz, whereas that of the h noise will stay below 150 Hz. This would mean that in the present recording conditions most of the high frequency m -noise components had no chance of appearing in the noise recordings. In consequence, the computed covariance and spectral

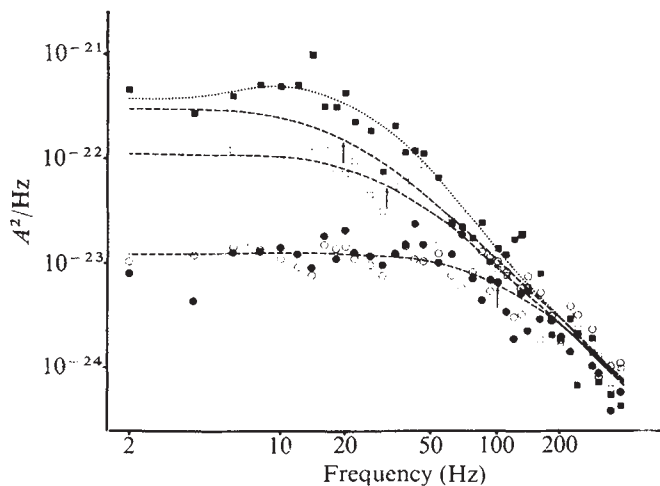


Fig. 3 Spectral density function (ordinate) computed for current noise recorded at resting potential (-70 mV) ($\bullet$) and at 13 mV ($\square$), and 14 mV ($\blacksquare$) depolarisation from resting potential, and at 16 mV depolarisation in the presence of 120 nM tetrodotoxin ($\circ$). ---, Theoretical values which are proportional to $[1 + (f^2/f_0^2)]^{-1}$, where f represents the frequency (abscissa) and f_0 the half-power frequency whose value varies between 20 and 100 Hz (arrows). The dotted line is fitted by eye.

density function can contain no information regarding the kinetics of the m system. The presence of low frequency m -noise components in the recorded noise may, however, explain the steep increase of the noise variance with increasing depolarisation. With respect to the h -noise, because of its narrow bandwidth it would be expected to pass the filtering circuits of the recording system fairly undistorted and therefore be able to reveal the kinetics of the h system. For this reason it is held that the voltage dependent changes in time constant of the covariance function,

or half-power frequency of the spectral density function are true indications of the voltage dependence of the kinetics of the h system. It is therefore concluded that in our preparation the time constant of the h system is increasing monotonously as the membrane potential is lowered towards firing threshold. This indicates a low degree of Na inactivation in near-threshold conditions. The reason for this could be an especially fast transition from 'closed' to 'open' conformation of the h system, as is occasionally found in particularly non-accommodating nerve fibres^{10,11}.

Functionally, such setting of the Na channel gating system would provide for the occurrence of a subthreshold steady-state Na conductance¹², which might explain the non-adapting mode of firing in our preparation¹³. This hypothesis is corroborated by the fact that a distinct Na noise is not demonstrable in the fast adapting stretch receptor neurone of lobster.

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Subthreshold membrane currents in slowly adapting stretch receptor neurone of lobster

IN the slowly adapting stretch receptor neurone of lobster, application of tetrodotoxin (TTX) causes a simultaneous hyperpolarisation and decrease in input resistance¹. These effects have been attributed to a possible increase in potassium conductance. They could, however, be explained by elimination of a subthreshold steady-state sodium conductance which, in this case, might underlie the cell's repetitive mode of firing². To examine this possibility, we made a voltage clamp study of the subthreshold steady-state membrane currents of the slowly adapting stretch receptor neurone of lobster. The results indicate the existence of a subthreshold steady-state Na current which to a large extent seems to be balanced by a leak type current.

We used isolated cells mounted in a chamber continuously perfused with solutions of desired composition at 18°C . Membrane potential and membrane currents were recorded under voltage clamp using a single, low resistance (5 – $8\text{ M}\Omega$) intrasomal microelectrode filled with 3 M KCl (ref. 3). This method permitted measurement of steady-state membrane currents that did not exceed 5 nA . Such currents were encountered in a voltage range of 10 – 15 mV in either direction from resting potential (Fig. 1). Steady-state values of the membrane current were determined 10 – 15 s after each polarisation adjustment.

Typical current measurements in the absence and presence of TTX (Fig. 1) show that in control conditions the curve relating membrane current to membrane polarisation had a negative slope starting at depolarisations of 5 – 7 mV . After treatment with 120 nM TTX the current-voltage curve lost this negative slope and became steeper. At the same time, its voltage intercept was shifted in a

negative direction by about 5 mV. In agreement with earlier findings these changes imply a hyperpolarisation as well as a decrease in input resistance in the absence of any injected current.

To see whether these changes originated only in the elimination of a Na conductance, all extracellular Na^+ was replaced by either choline or Tris. In both cases a current-voltage curve was obtained very similar to that encountered after TTX treatment, although potentials were more negative by about 5 mV. This curve underwent no further changes if TTX was added to the Na-free solutions. Thus TTX did not affect the K channel or the leak channel in our preparation and so its effect on the current-voltage relationship, as described above, must have been due to elimination of a specific Na conductance.

For an estimate of the magnitude of this conductance, the current-voltage curves obtained in the absence and presence of TTX, respectively, were subtracted from each other. The resulting relationship between Na current and membrane polarisation (Fig. 1) shows that Na conductance was appreciable in resting conditions and that it did not vanish until the cell was hyperpolarised by at least 10 mV; in depolarising conditions (down to firing threshold) the Na conductance increased at an increasing rate.

To study the nature of the membrane current, I_x , which balances the Na current in resting conditions, cells were exposed to 20 mM tetraethylammonium (TEA) after pre-treatment with 120 nM TTX. In the absence of any injected current this led to a slight depolarisation (2–3 mV) and a minor increase (10–20%) in the input resistance. These changes show—presuming that TEA eliminated the K conductance completely—that in resting conditions only 20–30% of I_x can be attributed to a specific K current. The remaining and greater part of I_x therefore seems to be a leak type current. The ions carrying this current presumably include not only K^+ and Cl^- but also Na^+ . The presence of a Na component in the leak current is suggested by the 5 mV hyperpolarisation that arises in response to removal of all extracellular Na^+ after preceding blockage of the specific Na channels by means of TTX.

Studies of membrane noise³ have suggested that the slowly adapting stretch receptor neurone of lobster has a small tendency for Na inactivation in subthreshold conditions. This type of permeability regulation probably explains the presence of a subthreshold steady-state Na conductance. The functional significance of such a conductance may be twofold. First, as it increases with depolarisation it increases the input resistance of the cell and thus reduces the load on the generator current source. Second, owing to its existence,

Fig. 1 Relationship between membrane current (ordinate) and membrane polarisation (relative to resting potential at -60 mV, abscissa) in the absence (∇), and presence ($\bullet$) of 120 nM TTX. The relationship between Na current and membrane polarisation (—) was obtained by subtracting the curves I_x and I_m from each other.

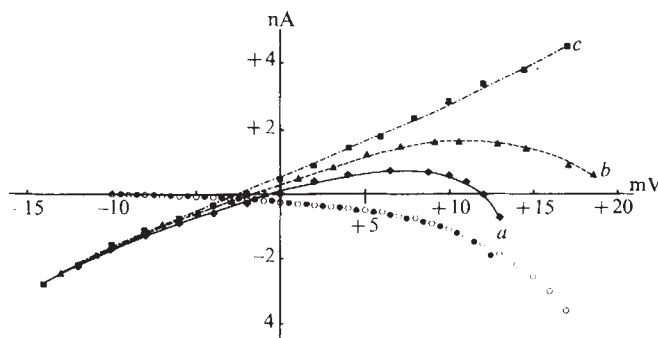
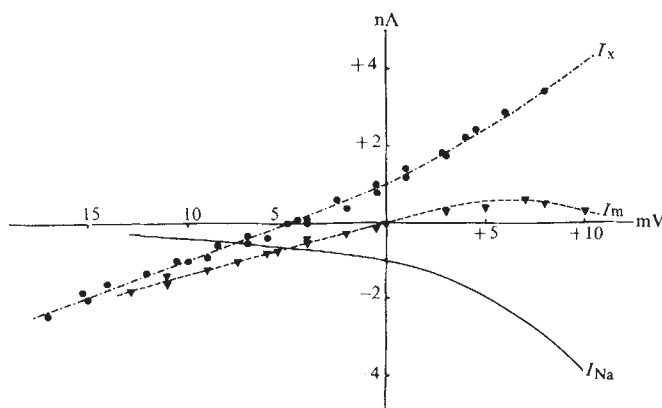


Fig. 2 Relationship between membrane current (ordinate) and membrane polarisation (relative to resting potential at -62 mV, abscissa) in control conditions ($\diamond$) and after application of TTX to the axon beyond 300–400 μm ($\blacktriangle$), and beyond 100 μm ($\blacksquare$) from the cell body. $\bullet$, Difference between curves a and b; $\circ$, difference between curves b and c.

conditions may be fulfilled for the repetitive mode of firing² that is typical of the preparation we used. There is evidence for this latter assumption in the fact that no subthreshold steady-state Na conductance exists in the fast adapting stretch receptor neurone of lobster.

In an attempt to localise the site of origin of the intrasomally detectable subthreshold steady-state Na current, TTX was applied to the axon at various distances from the cell body. As a rule, this resulted in a decrease of the inward component of the membrane current (Fig. 2), indicating that a subthreshold steady-state Na current is generated in the initial parts of the axon as well as in the soma. There was a considerable decrease in inward current even when TTX was applied to relatively distant axonal segments, as shown in Fig. 2. Application of TTX to the axon 300–400 μm from the cell body and beyond gave a marked decrease in inward current. This decrease equalled the further reduction of inward current obtained when extending the treatment about 200 μm towards the cell body. Considering the cable properties of the axon this means that the axonal segments beyond a distance of 300–400 μm from the cell body could generate a stronger inward current than more proximal axonal segments. This may be related to the presence of a spike-trigger zone with particularly developed pacemaker properties about 300–500 μm out on the axon^{4,5}.

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Plasma membrane-associated increase in guanylate cyclase activity in regenerating rat liver

CYCLIC GMP has been postulated as one of the intracellular mediators triggering cell growth¹. Exogenously added cyclic GMP or its derivatives are liable to induce a substantial increase in DNA synthesis^{2–4} and to promote growth^{2,5}. Cultured, non-transformed fibroblasts⁶ and lymphocytes¹ exist in one of two reversible growth states, a state of rapid

proliferation and a state of relative quiescence and increased levels of cyclic GMP are correlated with the transition from the resting to the growth state^{1,3}. In these systems, a variety of mitogenic agents have been shown to initiate proliferation of quiescent cells and this effect seems to be accomplished by interaction with the cell surface¹. Therefore, if an increase in cyclic GMP levels were to act as the intracellular signal triggering cell proliferation, its formation and/or degradation should be linked to events occurring in the plasma membrane. Indeed, a membrane-bound guanylate cyclase (GTP pyrophosphatase, cyclising, EC 4.6.1.2.) of cultured fibroblasts has been found to be activated by a fibroblast growth factor⁷.

Partial hepatectomy induces the remaining liver cells to undergo a change from a resting to a proliferating state similar to the one cited above for cultured cells⁸, and the regenerating liver after partial hepatectomy presents a well defined model system for studying cell proliferation *in vivo*. In the normal hepatocyte, however, most of the guanylate cyclase activity is recovered in the cytosol⁹. We therefore examined the possibility that an altered subcellular distribution of guanylate cyclase might be found in proliferating liver cells. Data presented show an increase in guanylate cyclase activity in liver homogenates after partial hepatectomy, mainly due to an increase in plasma membrane-associated activity.

We measured cyclic GMP formation in the total homogenate (for details of preparation, see Fig. 1) of regenerating rat liver at different times after partial hepatectomy. An increase in guanylate cyclase activity was observed within 16 h, reaching a maximum 24 h after the operation (Fig. 1). This increase in guanylate cyclase activity correlates with the increase in proliferating activity⁸. At 4 d after hepatectomy, when the regeneration is nearing completion, the elevated cyclic GMP formation returned to control values.

In the quiescent liver, guanylate cyclase is mainly detectable in the soluble fraction (Table 1). At 3 d after partial hepatectomy, the activity of the soluble guanylate cyclase did not change, whereas the plasma membrane-associated enzyme activity rose to about 11 times the value of sham-operated rats. Essentially the same changes were observed when the activities were expressed as pmol cyclic GMP formed per min per g liver (Table 1). Triton X-100 stimulates guanylate cyclase activity in a number of systems^{7,9,11}. Treatment of plasma membrane fractions from normal and regenerating liver with this detergent (Triton: protein = 2:1, w/w; 10 min at 0 °C) increased cyclic GMP formation 2.5–2.9 and 2.4–2.6-fold, respectively (three experiments). Thus, approximately the same increase in plasma membrane-associated guanylate cyclase activity was observed in the presence and absence of detergent. The technique used for the isolation of liver plasma membranes, aimed at obtaining the highest possible purity, makes losses of material inevitable. Using the recovery of 5'-nucleotidase (a marker enzyme for liver plasma membranes¹³) as reference, the yield of plasma membrane material in our preparations is about 11%. Table 1 indicates that more than 85% of the increased guanylate cyclase activity in regenerating liver should be localised in the surface membranes.

Rapidly growing tissue is found also in hepatoma tumours. In previous reports, no correlation between cyclic GMP levels and growth rate of hepatoma tumours was found, although cyclic GMP concentrations tended to be higher in hepatoma cells compared with normal liver^{14,15}. We examined guanylate cyclase activity in four hepatoma lines: Morris hepatomas 7777, 9618A₂, 7800 and 9121, the first two lines being rapidly-growing, the others medium-growing tumours. No detectable activity (less than 0.8 pmol cyclic GMP formed per min per mg protein) was found in three lines and an increased activity with respect to normal

liver in line 9121 (25.6 ± 2.8 pmol per min per mg protein; three experiments). Treatment with Triton X-100 did not elicit detectable activity in 7800, 9618A₂ and 7777 lines, although cyclic GMP formation was moderately stimulated in 9121 line (1.6-fold). Addition of hepatoma homogenate without detectable guanylate cyclase activity to normal liver or to the 9121 line did not reveal the presence of enzyme inhibitors. We postulate that a normally functioning guanylate cyclase is essential for growth regulation.

In young adult rats as used in the present experiments,

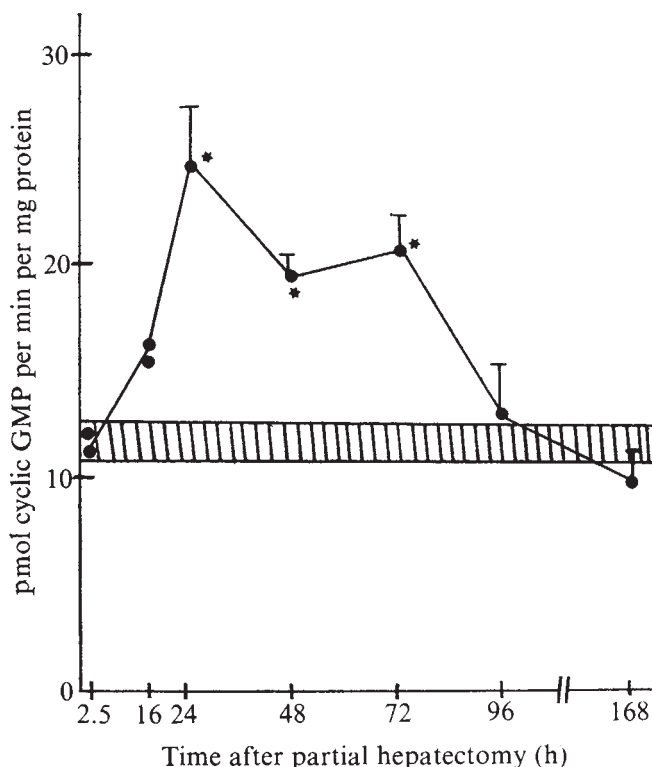


Fig. 1 Cyclic GMP formation in liver homogenates at various times after partial hepatectomy. Female Buffalo rats (150–200 g) were fed Altromin R, containing 19% protein, and water *ad libitum*. For partial hepatectomy two-thirds of the liver were removed. Sham-operated animals were used as controls. All surgical operations were carried out under ether anaesthesia in the morning between 0800 and 0900. For determinations of guanylate cyclase activity in the total homogenate, in the soluble and crude particulate fraction 0.5 g liver was homogenised in 3 volumes 10 mM Tris-HCl buffer, pH 7.4. Soluble and crude particulate fractions were obtained by centrifugation of the homogenate at 105,000g for 60 min. The pellet was washed once in the same buffer. Plasma membranes were prepared by a modified procedure of Neville's method¹⁰. The total homogenate and the soluble fraction could be stored for several weeks at –40 °C without loss of guanylate cyclase activity. The guanylate cyclase bound to the membrane, however, was highly unstable; the activity of a plasma membrane fraction stored for 2 d at –40 °C decreased by 50%. Rats were killed at the times indicated after partial hepatectomy and suitable dilutions (30–95 µg protein) of a 1:3 (w/v) liver homogenate assayed for guanylate cyclase activity as described previously¹¹ with two modifications: 0.1 mM papaverine was included in the reaction mixture to inhibit cyclic nucleotide phosphodiesterase activity; as rather low activities had to be measured in some fractions, the eluate of the Al₂O₃ columns was further chromatographed on Dowex 1 × 8 (formate form)¹². This step was very effective in lowering the blank values, which did not exceed 0.006% of the total radioactivity. Preliminary experiments showed that the MnCl₂ concentration used (8 mM) gave maximum activities in control and regenerating liver. Mean ± s.e.m. is given for 4 to 11 animals except for the 2.5 and 16-h values, where the figures for 2 animals are shown. Mean ± s.e.m. for 8 sham-operated rats is indicated by the horizontal bar. There were no differences between sham-operated animals killed 1–4 d after surgery. *Values significantly different from controls ($P < 0.005$).

Table 1 Cyclic GMP formation in various fractions derived from sham-operated and regenerating liver

	Guanylate cyclase activity			(pmol per min per g liver)		
	(pmol per min per mg protein) Sham operation	Partial hepatectomy	% increase	Sham operation	Partial hepatectomy	Increase*
Total homogenate	11.7 ± 0.7	20.8 ± 1.7	77	1,567 ± 81	2,892 ± 221‡	1,325
105,000g supernatant	28.9 ± 4.0	30.0 ± 2.4	—	1,879 ± 198	2,190 ± 168	—
105,000g pellet	3.6 ± 0.7	19.8 ± 4.5†	450	182 ± 27	1,439 ± 246†	1,252
Plasma membrane fraction	12.1 ± 0.8	141 ± 26‡	1,065	9.4 ± 0.6	135 ± 21‡	126

* Increase in guanylate cyclase activity expressed as pmol cyclic GMP per min per g liver.

Rats were killed 72 h after partial hepatectomy. This time was chosen to minimise possible nonspecific effects of surgery. The plasma membrane fraction was comprised of fractions M₁ and M₂ from modified Neville's¹⁰ method. Aliquots of a liver homogenate and of the various fractions which were in 10 mM Tris-HCl buffer (pH 7.5) were assayed for guanylate cyclase activity. Three dilutions (30–95 µg protein for total homogenate and supernatant, 40–140 and 16–100 µg for the 105,000g pellet and the plasma membrane fraction, respectively) of all fractions were tested to ensure that the reaction velocity was proportional to enzyme concentration. No measurable hydrolysis of added cyclic GMP by phosphodiesterase occurred in any assay. Values are means ± s.e.m. for 4–11 animals. Difference significant † *P* < 0.001 (‡ *P* < 0.005) from sham-operated controls.

DNA synthesis starts to increase 12–15 h after partial hepatectomy, peak values being attained at 19–25 h (refs 8 and 16). Mitosis follows DNA synthesis by about 8–9 h (ref. 8) and it has been estimated that about 40% of the parenchymal cells divide between 30 and 40 m (ref. 16). Thus the timing of the observed increase in guanylate cyclase activity correlates well with the onset of proliferative activity after partial hepatectomy, indicating that the induction of liver cell proliferation is intimately connected with increased activity of this enzyme. Fast transient increases in cyclic GMP concentrations have been reported to occur in several *in vitro* systems^{1,3,7} after administration of mitogenic agents, which presumably interact solely with the cell surface. The fact that the increment is predominantly, if not exclusively, located in the plasma membrane also indicates that in the regenerating liver cyclic GMP acts as signal to mediate growth-initiating signals¹⁷ from the cell surface to intracellular structures (for example, the nucleus). In agreement with this hypothesis, a guanylate cyclase associated with a plasma membrane fraction of mouse fibroblasts was found to be activated by a fibroblast growth factor⁷.

During the preparation of this manuscript, Kimura and Murad¹⁸ reported on an increase in particulate guanylate cyclase activity in regenerating liver. These authors describe a less than twofold increase in guanylate cyclase activity in a plasma membrane fraction of regenerating rat liver compared with the more than 11-fold increment observed here; and we cannot confirm a decrease in the soluble enzyme activity during liver regeneration. Kimura and Murad measured an increased enzyme activity in Morris hepatoma 3924A. Using Morris hepatoma 9121 we also measured increased guanylate cyclase activity, but in three other lines (7777, 7800, and 9618A₃) no enzyme activity could be detected. These contradictory findings in Morris hepatomas demonstrate that no simple correlation between proliferation of normal tissue and malignant growth is possible.

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Increases in cyclic GMP levels in brain and liver with sodium azide an activator of guanylate cyclase

SODIUM azide produces extrapyramidal symptoms with necrosis of the cerebral cortex, cerebellum, and basal ganglia. Toxicity with this metabolic inhibitor may also include hypotension, blindness and hepatic necrosis^{1–5}. Many hormones and drugs mediate their effects by altering the intracellular levels of cyclic AMP and/or cyclic GMP^{6–10}. We found that NaN₃ increases cyclic GMP levels in incubations of slices from cerebral cortex, cerebellum or liver, and also stimulates guanylate cyclase activity. In contrast to other agents that can increase cyclic GMP accumulation in tissues the effect of NaN₃ is observed in the absence of added calcium.

In slices from cerebral cortex, cerebellum and liver cyclic GMP levels increased within 5 s after the addition of 1 mM NaN₃ and, in 1–5 min, reached maximal levels (two to fivefold increases) that were maintained for 10 min (Fig. 1). With 1 mM NaN₃ there was no change in the level of cyclic AMP in these tissues at any of the times (5 s–10 min) examined. The accumulation of cyclic GMP in cerebral cortex slices depended on the concentration of NaN₃. NaN₃ (0.15 mM) produced a half-maximal effect and 1 mM NaN₃ was maximally effective. NaCN, another metabolic inhibitor, had little or no effect on cyclic GMP or cyclic AMP levels in cerebral cortex incubations (Table 1). Atropine (0.01 mM), (±)-propranolol (0.01 mM),

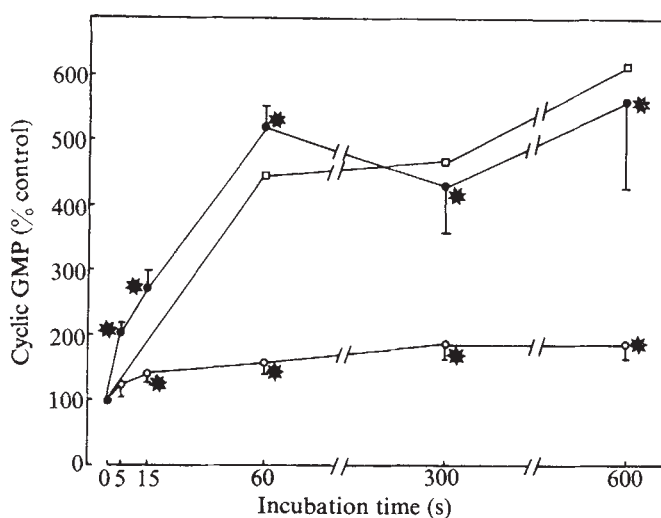


Fig. 1 Effect of NaN_3 on cyclic GMP levels in slices of cerebral cortex (●), cerebellum (○) and liver (□). Male Sprague-Dawley rats (150–200 g) were decapitated and slices of cerebral cortex, cerebellum and liver were incubated at 37 °C for 60–90 min in Krebs–Ringer bicarbonate (KRB) medium with 95% O_2 and 5% CO_2 . Slices were washed with fresh medium and incubated with 1 mM NaN_3 for the times indicated. Incubations were terminated by addition of 5% TCA and homogenised. Cyclic GMP and cyclic AMP were purified and assayed as described previously^{11–13}. Guanylate cyclase activity was determined as described previously¹³. Protein was estimated by the method of Lowry *et al.*¹⁴. Values presented are means from 2 to 10 incubations and are expressed as % control. Vertical bars indicate 1 s.e. Control values for cyclic GMP are 0.41 ± 0.09 ($n = 10$), 14.5 ± 2.5 ($n = 10$) and 0.06 ($n = 2$) pmol per mg protein for cerebral cortex, cerebellum and liver, respectively. * $P < 0.05$.

phenoxybenzamine (0.012 mM), diphenhydramine (0.1 mM), and γ -aminobutyric acid (1 mM) had no effect on cyclic GMP or cyclic AMP levels in cerebral cortex or cerebellum incubations with or without 1 mM NaN_3 . Hydroxylamine (0.1 mM), an agent that increases the concentration of γ -aminobutyric acid in brain⁶, markedly increased cyclic GMP levels in slices of cerebral cortex (Table 1). Aminooxyacetic acid (1 mM) which increases γ -aminobutyric acid levels in brain⁶, had no effect, however, on cyclic GMP or cyclic AMP levels in incubations of cerebral cortex slices with or without 1 mM NaN_3 .

The stimulatory effects of NaN_3 and NH_2OH on cyclic GMP accumulation were additive (Table 1).

The accumulation of cyclic GMP in incubations of cerebral cortex slices with 1 mM NaN_3 was unaltered when calcium-free medium was used (Table 1). The increase in cyclic GMP levels with 80 mM KCl, a depolarising agent at this concentration, required the presence of Ca^{2+} . The presence or absence of 2.5 mM Ca^{2+} in the incubation medium had a small effect on basal cyclic GMP levels. These results indicate that there are at least two mechanisms to increase cyclic GMP levels in tissues—

Fig. 2 Effect of NaN_3 on soluble (○) and particulate (●) guanylate cyclase from cerebral cortex. Rat cerebral cortex was homogenised at 4 °C in 9 volumes 0.25 M sucrose containing 5 mM Tris-HCl buffer, pH 8.0, using a glass homogeniser with a Teflon pestle. Homogenates were filtered through gauze and centrifuged at 105,000g for 60 min to separate soluble and particulate fractions. Enzyme preparations (20–50 μg protein) were preincubated for 5 min at 37 °C in mixtures of 50 mM Tris-HCl buffer, pH 7.6, 10 mM theophylline, 15 mM creatine phosphate, 20 μg creatine phosphokinase (120–135 U mg^{-1}), with or without NaN_3 as indicated, in a final volume of 100 μl . Reactions were started by addition of 3 mM MnCl_2 and 1 mM GTP (final concentrations), incubated for 5 min and terminated by adding 0.9 ml 50 mM Na acetate buffer, pH 4.0, and heated for 3 min at 90 °C (ref. 13). Cyclic GMP formed was determined by radioimmunoassay. Values presented are means of duplicate determinations.

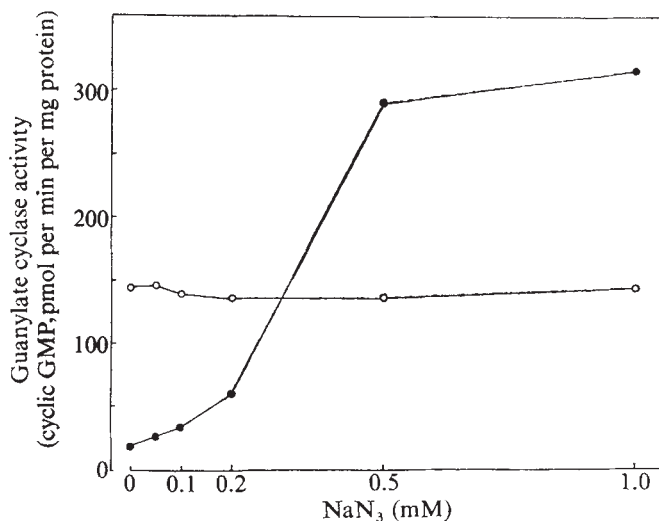


Table 1 Effects of NaN_3 and other compounds on cyclic nucleotide levels in slices of cerebral cortex in the presence and absence of Ca^{2+}

Addition	Incubation time (s)	Cyclic GMP (pmol per mg protein)		Cyclic AMP (pmol per mg protein)	
		KRB medium	Calcium-free medium	KRB medium	Calcium-free medium
None	—	0.77	—	2.85	—
NaCN (1mM)	60	1.01	—	3.03	—
None	—	0.41	—	3.74	—
NH_2OH (0.1 mM)	60	2.74	—	2.88	—
NaN_3 (1 mM)	60	1.78	—	3.48	—
NH_2OH (0.1 mM) + NaN_3 (1 mM)	60	4.21	—	2.88	—
None	—	0.16	0.24	2.42	3.19
NaN_3 (1 mM)	15	0.44	0.85	2.72	3.23
NaN_3 (1 mM)	60	1.03	2.08	2.77	3.07
None	—	0.19	0.54	1.28	1.53
KCl (80 mM)	60	0.39	0.56	1.58	1.64
KCl (80 mM)	300	2.75	0.28	1.72	1.82

Cerebral cortex slices were incubated, washed with calcium-free KRB medium with 0.1 mM EGTA, and incubated an additional 30 min before NaN_3 or other agents were added. Slices were incubated with compounds indicated in the presence and absence of Ca^{2+} . Incubations were terminated by addition of 5% TCA and homogenised. Cyclic nucleotides were determined as described previously^{11–13}. When media containing no Ca^{2+} or high K^+ were used, the concentration of Na^+ was changed to maintain the total concentration of cation at 150 mM. Values presented are means of duplicate incubations.

one calcium-dependent and the other apparently calcium-independent.

The soluble and particulate guanylate cyclase activities in tissues have different properties and can be separated with gel filtration techniques¹³. Soluble guanylate cyclase demonstrates typical Michaelis-Menten kinetics and is activated by calcium. Particulate guanylate cyclase, which is associated with plasma membranes, Golgi and endoplasmic reticulum, is an allosteric enzyme and inhibited by calcium¹³. Soluble and particulate guanylate cyclase in cerebral cortex were examined, and we obtained results similar to those reported for other tissues. The particulate enzyme from cerebral cortex was markedly stimulated by NaN_3 , whereas NaN_3 had no effect on soluble guanylate cyclase (Fig. 2). The stimulation of particulate guanylate cyclase depended on the concentration of NaN_3 , with half-maximal activation occurring with 0.3 mM NaN_3 , a concentration similar to that required with slices. NH_2OH was a more potent stimulator of the particulate enzyme, but not the soluble enzyme from cerebral cortex. Half-maximal activation was obtained at 7 μM NH_2OH . Activation of guanylate cyclase by NaN_3 did not result from the preservation of substrate (GTP) or the inhibition of cyclic nucleotide phosphodiesterase activity. Various halide ions and cyanide (1 mM) had no effect on either soluble or particulate guanylate cyclase.

The presence of two forms of guanylate cyclase¹³ in tissues in which one is activated and the other is inhibited by Ca^{2+} may be the explanation for our observations. Generally the inhibitory effects of NaN_3 with a variety of enzymes require higher concentrations than those used in this study¹⁶. Cyanide, another metabolic inhibitor, had small effects on cyclic GMP levels and guanylate cyclase activity. The effects of NaN_3 and NH_2OH are probably not due to general inhibition of intracellular metabolism. Although the effects of NaN_3 on guanylate cyclase can explain the increased accumulation of cyclic GMP observed with intact tissues, other effects of NaN_3 cannot be excluded. NaN_3 and NH_2OH are known to be strong nucleophilic substances. Additional studies are also needed to determine whether or not the toxic manifestations of NaN_3 are related to the stimulation of guanylate cyclase and increased cyclic GMP accumulation in tissues.

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Positive control of cyclic AMP on mesenchymal factor controlled DNA synthesis in embryonic pancreas

THERE is increasing evidence that cyclic AMP and/or cyclic GMP are involved in the regulation of a number of metabolic processes including cell proliferation¹⁻¹³. We report here the results of an investigation testing the effects of cyclic AMP, cyclic GMP and the mesenchymal factor (MF)¹⁴⁻¹⁷ on the stimulation of DNA synthesis in embryonic epithelia in organ culture.

The normal development of most organ systems requires the interaction of epithelial and mesenchymal cells^{18,19}. We have used the pancreas as a paradigm for the epithelial-mesenchymal interaction. A factor obtained from mesenchymal tissues or from mesenchyme-rich embryonic tissues can substitute for the mesenchyme to induce normal growth and development¹⁴⁻¹⁷. MF activity is sensitive to both trypsin¹⁵ and periodate oxidation (ref. 17, and S. Levine, R. P. and W. J. R., unpublished observations), suggesting that protein and carbohydrate moieties are required for its function. Purified MF covalently bound to Sepharose beads induces DNA synthesis in the pancreatic epithelial cells. The experimental results suggest that MF functions through an interaction with the cell surface^{16,17}. The effects of MF may therefore be mediated by secondary messengers such as cyclic AMP or cyclic GMP. We have therefore tested the effects of these compounds as well as

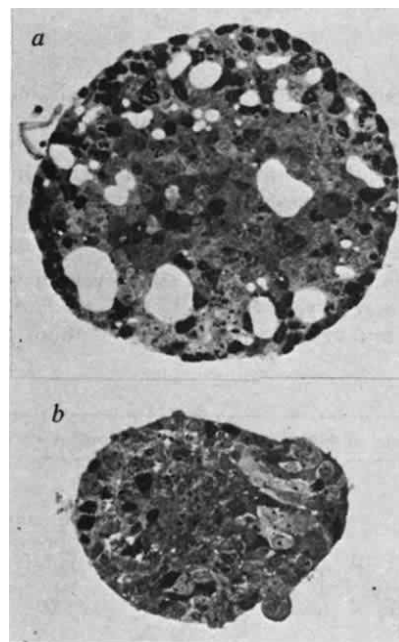


Fig. 1 The incorporation of ^3H -thymidine in the presence of inactivated MF and cyclic AMP is due to the labelling of many cells. Pancreatic epithelia were isolated and cultured as described in Table 1 in medium containing 1 mg ml⁻¹ of inactivated factor in the presence (a) or absence (b) of 10^{-3} M 8-OH-cyclic AMP. The epithelia were harvested and fixed in 4% glutaraldehyde in 0.04 M phosphate buffer (pH 7.4). After postfixation in osmium they were embedded in Epon²⁹. Thick sections were placed on the slides and coated with an Ilford L-4 emulsion³⁰ and developed after 10 d of exposure. After 90 min of treatment with sodium periodate MF is not totally deprived of activity: by increasing its concentration there is higher thymidine incorporation (see Fig. 2b) than is reflected by a few labelled cells in Fig. 1b. But the addition of 8-OH-cyclic AMP increases dramatically the number of cells which undergo DNA synthesis (Fig. 1a). The vacuoles observed are caused by the addition of cyclic AMP and probably reflect the increased fluid transport into the existing exocrine lumen which is already present at that time of development³¹.

their derivatives on the DNA synthesis of pancreatic epithelia (mesenchyme-free) in the presence and absence of MF. None of the compounds affected MF activity; but unexpectedly, MF which had been inactivated could be completely reactivated by inclusion of dibutyl cyclic AMP (db-cyclic AMP) or 8-OH-cyclic AMP though not by cyclic AMP, cyclic GMP or cyclic GMP analogues. The effects of 8-OH-cyclic AMP and 8-Br-cyclic GMP on DNA synthetic activity in the presence of MF or inactivated MF are summarised in Table 1. Neither 8-OH-cyclic AMP nor 8-Br-cyclic GMP stimulated or inhibited DNA synthesis when the epithelia were incubated in the presence of active MF. On the other hand, 8-OH-cyclic AMP but not 8-Br-cyclic GMP stimulated the activity of periodate-inactivated MF to the levels of incorporation observed with untreated MF. We have shown previously that in the presence of MF, ^3H -thymidine is incorporated into the DNA in many cells, whereas, in contrast, incubation with inactivated MF leads to ^3H -thymidine incorporation in very few cells¹⁷. Figure 1a and b demonstrates that the addition of 8-OH-cyclic AMP increases thymidine incorporation into cells in the presence of inactivated MF. Thus the effect of cyclic AMP is to increase the proportion of cells undergoing DNA synthesis. The specificity of this response is reported in Table 2. Whereas 8-OH-cyclic AMP and db-cyclic AMP stimulate DNA synthesis in the presence of inactivated MF, neither 5'-AMP nor cyclic AMP itself even when present with the phosphodiesterase inhibitor, caffeine, promote DNA synthesis. Some MF preparations that become inactive spontaneously can also be activated by db-cyclic AMP derivatives. The activation of DNA synthesis by db-cyclic AMP or 8-OH-cyclic AMP but not cyclic AMP is taken to mean that entrance into the cells is a requisite for activity. All attempts to reactivate the factor itself by treatment with cyclic AMP or cyclic AMP analogues before its addition to the cells have failed. For example, inactivated MF incubated for 20 h at 37 °C with 10^{-3} M db-cyclic AMP is inactive after removal of the free cyclic nucleotides by chromatography on Sephadex G-25, but stimulates DNA synthesis on addition of db-cyclic AMP into the culture medium. The incorporation of ^3H -thymidine into DNA is dependent on both the concentration of inactivated MF and 8-OH-cyclic AMP. As shown in Fig. 2a, increasing the concentration of inactive factor in the presence of 10^{-3} M 8-OH-cyclic AMP results in an increase of thymidine incorporation into DNA. In fact, the saturation profile is similar to that observed with active MF alone¹⁷. In the presence of a single concentration of MF (Fig. 2b), addition of 8-OH-cyclic AMP gives a typical hyperbolic activation profile (low levels of activity are always found with periodate inactivated MF preparations). The activation

Table 1 Inactivated MF stimulates DNA synthesis in the presence of cyclic AMP but not cyclic GMP

	8-OH-cyclic AMP (10^{-3} M)	8-Br-cyclic GMP (10^{-4} M)	^3H -Thymidine incorporated into DNA (c.p.m. per epithelium per 6 h)	<i>n</i>
Active MF	—	—	7,330 (5,200/9,800)	8
	+	—	8,680 (5,660/13,100)	6
	—	+	7,495 (6,700/10,000)	10
Inactivated MF	—	—	290 (97/420)	6
	+	—	6,500 (3,300/8,020)	8
	—	+	264 (180/405)	10

Inactivated MF stimulates DNA synthesis in the presence of cyclic AMP but not cyclic GMP. Pancreatic epithelia were isolated from rat embryos on day 12 of gestation (about 35 somites) as previously described¹⁴⁻¹⁷. Individual epithelia were transferred in Dispo tray wells (Linbro, Los Angeles) and cultured for 18 h in 100 μl of medium (Eagle's basal medium in which the essential amino acids have been increased sevenfold ($7 \times \text{BME}$), plus 1 mg ml^{-1} bovine serum albumin, 1 μg , ml^{-1} penicillin, 10^{-4} mg ml^{-1} streptomycin and 25 μg ml^{-1} fungisone) in an atmosphere of 5% CO_2 in air. The nucleotides were dissolved in the $7 \times \text{BME}$; and 100 μl of this solution was added per ml of culture medium. Dilutions were made from a 10^{-2} M stock solution which was kept frozen at -70°C . Inactivated MF or MF was present at a final concentration of 600 μg protein ml^{-1} . For assays of DNA synthesis the epithelia were further incubated for 6 h in fresh identical culture medium containing ^3H -thymidine (10 μCi ml^{-1}). At the end of this period the epithelia were harvested, washed three times with EBSS containing 0.1% albumin, transferred into microfuge tubes (Beckman) and frozen. To measure the thymidine incorporated 100 μl 0.1% SDS was added and the tissue was disrupted by sonication at 0°C by rubbing the probe of the sonicator along the tube for 30 s. Aliquots (40 μl) of the suspension were spotted on GF Whatman filters. The filters were washed 6 times with cold 5% TCA, 3 times with 95% ethanol and twice with ether. The filters were dried and counted in scintillation fluid (100 ml NCS, 16 ml H_2O , 15.3 g Omnifluor added to 3.8 l of toluene). Results are expressed as c.p.m. ^3H -incorporated per epithelium during the 6-h incubation in the presence of ^3H -thymidine. MF was prepared as described¹⁶. For oxidation with sodium periodate, MF in 0.8 M KCl, 0.01 M Tris-HCl buffer (pH 8.0) was dialysed against 0.1 M glycine buffer at pH 8.0. After being dissolved in the same buffer sodium periodate was added at a final concentration of 50 mM. The reaction was allowed to proceed at 0°C in the dark for 90 min. To stop the oxidation tenfold molar excess of glycerol was added. After 15 min the periodate-treated MF (IO_4MF) was dialysed against 0.1 M glycine buffer (pH 8) followed by dialysis against 0.1 M glycine (pH 9). The preparation was then treated with sodium borohydride, dissolved in 0.1 M NaOH and added at a final concentration of 0.1 M. The preparation ($\text{IO}_4\text{BH}_4\text{MF}$) was dialysed against 0.01 M glycine buffer (pH 9). This fraction is referred to as inactivated MF. Identical results have been obtained if IO_4MF is not reduced with sodium borohydride. The effect of the oxidation seems specific: control experiments (S. Levine, R.P., and W.J.R., unpublished) show the sodium periodate is not toxic by itself since simultaneous addition of IO_4Na and glycerol have no effect on MF activity no more than direct reduction of MF by NaBH_4 . The inactivation by oxidation does not result from activation of an inhibitor since mixing of MF and IO_4MF does not decrease the activity of MF. The numbers in parentheses represent the lowest and highest values. *n* indicates the number of epithelia assayed.

Fig. 2 The degree of stimulation of DNA synthesis is dependent on the concentration of both inactivated MF and the cyclic AMP derivative. Pancreatic epithelia were isolated, cultured and DNA synthesis measured as described in Table 1. *a*, 9 epithelia were cultured in the presence of various concentrations of inactivated factor in the presence (●) or absence (○) of 10^{-3} M 8-OH-cyclic AMP. Periodate borohydride treated MF was concentrated up to 14 mg ml^{-1} in an Amicon stirred cells (Amicon Corp., Lexington, Massachusetts) using a PM-10 filter. The intermediate concentrations were obtained by dilution from that stock solution and added as usual at 100 μl ml^{-1} . The increase of thymidine incorporation in epithelia cultured in the presence of concentrated inactivated MF is due to the fact that after 90 min oxidation there is some remaining activity. *b*, Sets of 9 epithelia were cultured in the presence of 300 μg ml^{-1} of inactivated MF and various concentrations of 8-OH-cyclic AMP. The bars represent the s.e.m. of the tritium counts.

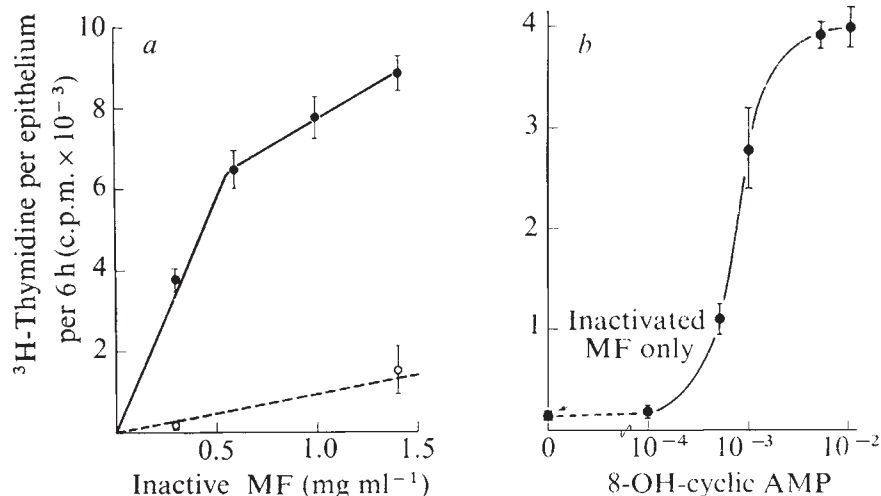


Table 2 DNA synthetic activity in the presence of inactive MF is dependent on cyclic AMP

	Nucleotides (10^{-3} M)	^3H -Thymidine incorporation into DNA (c.p.m. per epithelium per 6 h)	<i>n</i>
Inactivated MF	—	160 (128/190)	9
Inactivated MF	db-cyclic AMP	3,009 (870/5,630)	8
Inactivated MF	8-OH-cyclic AMP	3,370 (2,230/4,040)	9
Inactivated MF	Cyclic AMP	139 (110/190)	9
Inactivated MF	Cyclic AMP + caffeine (10^{-2} M)	267 (91/380)	9
Inactivated MF	5'-AMP	139 (115/390)	9
Inactive MF	—	205 (116/392)	5
Inactive MF	db-cyclic AMP	9,361 (7,625/10,795)	6

DNA synthetic activity in the presence of inactive MF is cyclic AMP dependent. Experiments were carried out as described in the legend of Table 1. Inactivated MF was present at a concentration in the incubation medium of $300 \mu\text{g ml}^{-1}$ and inactive MF at $600 \mu\text{g ml}^{-1}$. Inactive MF refers to MF preparations which spontaneously lose activity upon storage at 0°C . In such conditions, MF may lose its activity in a few days, but such inactive MF as well as IO_4MF or $\text{IO}_4\text{BH}_4\text{MF}$ are reactivated by cyclic AMP derivatives even after weeks of storage at 0°C . The numbers in parentheses represent the lowest and highest values. *n* represents the number of epithelia assayed

profile of db-cyclic AMP is different: a peak of activity is reached between 5×10^{-3} and 10^{-3} M. At higher concentrations inhibition is observed, perhaps because of the accumulation of toxic levels of butyrate.

As shown in Table 3, inactive MF is required for the activation of DNA synthesis. Cyclic GMP or cyclic GMP derivatives do not stimulate DNA synthesis by themselves. Furthermore, addition of varying concentrations of 8-Br-cyclic GMP in the presence of optimal levels of 8-OH-cyclic AMP do not induce DNA synthesis in the absence of inactivated MF (Table 3). These experiments give no evidence for a role of cyclic GMP as an intermediate in the action of MF.

Several processes that are dependent on cyclic AMP have been shown to require or involve calcium²⁰. In these systems calcium ionophores frequently influence the process²¹⁻²⁴. We have therefore investigated the effect of the calcium ionophore A23187 on DNA synthesis in the pancreatic epithelia. This compound at levels between 10^{-7} and 10^{-6} M does not stimulate the incorporation of ^3H -thymidine into DNA. In fact, at the highest concentrations tested (4×10^{-7} and 10^{-6} M) there is inhibition of the usual stimulation produced by inactive MF and 8-OH-cyclic AMP. We consider the experiments with calcium ionophore equivocal because these compounds allow transport of other ions and are present over relatively long periods of time (24 h) during which the stimulation of incorporation of precursors into DNA is observed.

The results of the present investigations suggest that our mesenchymal factor preparations contain two functional activities. This may explain our previous difficulties in

purification of MF and suggests that assays in the presence of 8-OH-cyclic AMP may detect one component, and that assays in the presence of inactive factor detect another function. The results of the present experiments suggest that one function, perhaps requiring a carbohydrate moiety (because of the sensitivity to periodate oxidation), may act through the enhancement of intracellular levels of cyclic AMP. The evidence for this is admittedly indirect, since intracellular levels of cyclic AMP have not been measured. The specificity of the response for cyclic AMP derivatives which penetrate cells and the magnitude of the response are, however, compatible with this view. The mode of action of cyclic AMP in this system is not yet resolved and an indirect effect of cyclic AMP is possible: intracellular cyclic AMP may enhance the responsiveness of the cell to 'inactive factor' by, for example, increasing the density of appropriate receptors on the cell membrane.

It is somewhat unexpected that the cyclic AMP analogues have a positive effect on DNA synthesis. In other experiments involving cells in culture, addition of compounds which increase intracellular cyclic AMP levels inhibit DNA synthesis¹⁻⁴. Furthermore, in a variety of cells there seems to be an inverse correlation between the level of intracellular cyclic AMP and DNA synthesis⁶⁻⁹. There seems also to be a positive correlation between increased cyclic GMP levels and DNA synthesis in some systems¹⁰⁻¹³. These results contrast with the findings reported here. Cyclic AMP is required for DNA synthesis; cyclic GMP or its derivatives have no effect by themselves, and do not inhibit the cyclic AMP response. It is possible that secretory cells whose secretory functions are positively regulated by cyclic AMP may also develop under the positive control of cyclic AMP. The positive control by cyclic AMP is analogous to that inferred in the action of a number of other peptide hormones²⁵⁻²⁸ which apparently act through cyclic AMP. The response observed in the embryonic pancreatic cells may also represent a characteristic of differentiating systems, that is, that high levels of cyclic AMP are required for a differentiative transition. Thus, proliferation of cells undergoing differentiation may have a different regulatory mechanism than the various cell types normally studied in cell culture. The developing pancreatic epithelium may be useful to define the role of cyclic AMP on the regulation of DNA synthesis and cytodifferentiation.

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Table 3 Addition of cyclic GMP does not influence DNA synthesis in embryonic pancreatic cells

Protein added to medium	Addition					
	None	8-OH-cyclic AMP (10^{-3} M) + 8-Br-cyclic GMP (M)				
		—	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Inactivated MF	205 (140/330)	1,414 (751/2,112)	2,025 (1,540/2,500)	2,098 (1,230/2,420)	1,978 (650/2,980)	2,452 (1,630/3,100)
Albumin	144 (106/195)	162 (91/233)	178 (71/362)	213 (143/501)	247 (107/552)	303 (210/403)

Addition of cyclic GMP or its derivatives does not influence DNA synthesis of embryonic pancreatic cells. Experiments were carried out in the same way and conditions are as described in the legend for Table 1. Inactivated MF was added at a concentration of about $300 \mu\text{g ml}^{-1}$ and albumin at 1 mg ml^{-1} . The addition of db-cyclic GMP or cyclic GMP in place of 8-Br-cyclic GMP led to identical results. The numbers are the average of 8 or 9 epithelia; the numbers in parentheses correspond to the lowest and highest values.

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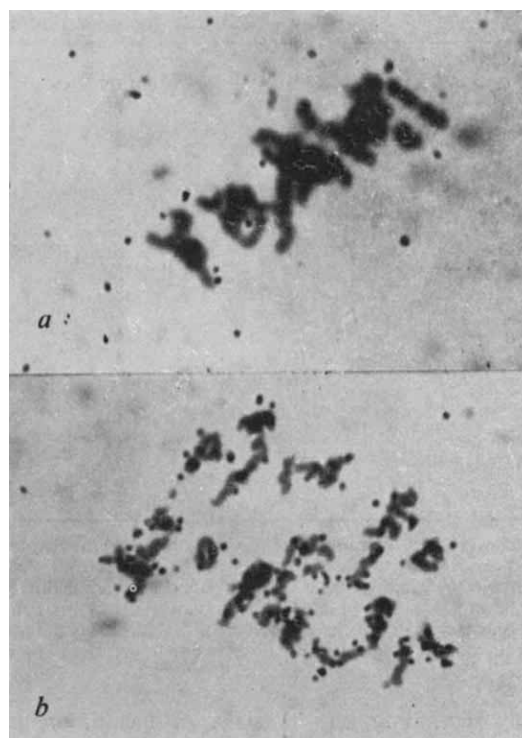


Fig. 1 Unscheduled DNA synthesis in mouse oocytes. *a*, MII control ($\times 1,150$). *b*, MII irradiated (60 J m^{-2} ; $\times 800$). Oocytes were irradiated in isotonic PBS containing 2 mg ml^{-1} polyvinyl pyrrolidone at 2–18 h after isolation using germicidal low pressure mercury discharge lamps (dose rate $1.3 \text{ J m}^{-2} \text{ s}^{-1}$) and immediately incubated in medium containing ^3H -thymidine ($20 \mu\text{Ci ml}^{-1}$; specific activity 40 Ci mmol^{-1}) for 1 h and cultured for 1 h in unlabelled medium. Non-irradiated oocytes served as controls. After washing, all oocytes were fixed (ethyl alcohol-glacial acetic acid, 3:1), placed on microscope slides, and incubated at 37°C in an atmosphere of 45% acetic acid for 5–10 min and air dried. Before dipping the slides in Kodak NTB emulsion, they were soaked in 5% trichloroacetic acid at 4°C for 30 min, washed in 95% ethanol and air dried. Exposure to the emulsion was for 2 weeks.

Ultraviolet light-induced unscheduled DNA synthesis in mouse oocytes during meiotic maturation

ULTRAVIOLET light-induced incorporation of ^3H -thymidine into DNA by non-S-phase nuclei has been observed in cultured mammalian cells¹, spermatogenic cells^{2–4}, and embryos⁵. This unscheduled DNA synthesis is generally considered to be the result of excision and replacement of ultraviolet-damaged bases by repair enzymes^{6–8}. We have examined the repair capacity of full-grown mouse oocytes after exposure to ultraviolet irradiation. As sensitivity to chemical mutagens and X irradiation changes during meiotic maturation in many species, we determined whether the ability to repair damage to DNA changes during mouse oocyte maturation *in vitro*. Oocytes were found to be capable of unscheduled DNA synthesis during meiotic maturation; however, irradiation of germinal vesicle stage oocytes induced higher levels of ^3H -thymidine incorporation than irradiation at metaphase I or metaphase II stages.

Fully-grown oocytes aseptically isolated from the ovary of adult mice were cultured in chemically defined medium⁹. Oocytes were irradiated 2–18 h after isolation using germicidal lamps, and incubated immediately in medium containing ^3H -thymidine. Non-irradiated oocytes served as controls. Incorporation of ^3H -thymidine into chromosomes was studied by autoradiography.

The progression of meiotic maturation of the oocytes during the culture period is summarised in Table 1. Many of the oocytes underwent germinal vesicle (GV) breakdown within 3 h of isolation. Metaphase I (MI) was reached by 4–8 h after the start of culture. Chromosomes at this stage were short and thick and were usually recognised as pairs of homologues. Oocytes cultured for 16–18 h reached second metaphase (MII), as evidenced by appearance of the first polar body (PBI). Oocyte chromosomes at MII were more clustered than those at MI. Whereas the morphology of the MII chromosomes was

well preserved (Fig. 1*a*), PBI chromosomes deteriorated, with bridge formation and fusion, into a mass of chromatin.

Autoradiography showed that grain counts over the GV or chromosome set were significantly higher (Student's *t* test, $P < 0.001$) after ultraviolet irradiation than in controls, regardless of the stage of meiotic maturation at which they were irradiated (Table 2, Fig. 1*b*). The corrected oocyte grain count increased with dose at 30 and 60 J m^{-2} but, except for the MI oocyte, there was no further increase in grain count at 120 J m^{-2} . Irradiated PBI had significantly higher observed and corrected grain counts than controls at a dose of 30 J m^{-2} ($P < 0.001$), approximately half that localised over MI or MII chromosomes; but corrected grain counts for the PBI were not significantly above controls when higher doses were given, suggesting that the slight repair capacity of the PBI was lost, perhaps by ultraviolet disruption of polar body integrity.

Grain counts over ultraviolet-irradiated oocytes differed

Table 1 Progression of meiotic maturation in mouse oocytes in culture

Time in culture (h)	No. of oocytes	% Oocytes		
		GV	MI	MI
2–4	108	33	67	0
6–8	12	0	100	0
16–18	120	7	15	78

Fully grown oocytes were isolated aseptically from the ovaries of 8–10-week-old mice (DUB/ICR strain, Flow, Dublin, Virginia) and cultured at 37°C with an atmosphere of 5% CO_2 –95% air in oocyte culture medium⁹.

Table 2 Incorporation of ^3H -thymidine into chromosomes of oocytes

Stage of oocytes	Dose (J m^{-2})	No. of oocytes	Grain counts on GV and chromosomes	
			Observed grains (mean $\pm$ s.e.)	Corrected for background grains (mean $\pm$ s.e.)
GV	0	13	19.6 $\pm$ 7.0	3.8 $\pm$ 6.0
	30	4	182.3 $\pm$ 90.7	126.8 $\pm$ 80.6
	60	13	694.5 $\pm$ 133.0	605.4 $\pm$ 114.6
	120	16	600.9 $\pm$ 155.8	576.7 $\pm$ 149.0
MI	0	23	2.2 $\pm$ 0.8	-4.2 $\pm$ 1.9
	30	8	34.1 $\pm$ 8.9	37.2 $\pm$ 11.7
	60	32	65.4 $\pm$ 10.1	76.7 $\pm$ 11.2
	120	38	83.9 $\pm$ 10.7	89.6 $\pm$ 12.7
MII	0	16	4.9 $\pm$ 2.4	3.2 $\pm$ 1.8
	30	11	30.0 $\pm$ 5.5	29.7 $\pm$ 5.8
	60	11	43.9 $\pm$ 9.9	37.0 $\pm$ 11.3
	120	15	39.3 $\pm$ 6.5	39.5 $\pm$ 7.4
PBI	0	21	2.5 $\pm$ 0.5	-2.2 $\pm$ 2.1
	30	12	18.8 $\pm$ 6.7	15.8 $\pm$ 7.4
	60	49	10.3 $\pm$ 1.7	9.7 $\pm$ 2.2
	120	12	11.4 $\pm$ 3.3	7.6 $\pm$ 4.0

Oocytes were treated as in Fig. 1. Grains on autoradiographs were counted using an ocular micrometer sectioned in $100 \mu\text{m}^2$ areas. Number of grains observed over chromosomes or GV was scored. As the grain density of background as well as the area of the chromosomes or GV varied, however, grain number was corrected for background by the following formula: corrected grain counts = $(A - N)(B/n)$, where N is the number of unit areas in which all chromosomes or GV is confined and A is the total grain number in N areas, n is the number of background unit areas adjacent to the chromosomes or GV, and B is the total grain count in n areas. This correction gave negative values for control oocytes when grain counts over chromosomes were lower than the average background counts.

markedly depending on the stage of meiotic maturation, whereas those of control oocytes invariably remained low (Table 2, Fig. 2). There was a large number of grains on GVs of irradiated oocytes, whereas fewer grains were localised over MI and MII chromosomes, and fewer still over polar body chromosomes. Because of the intervening meiotic reduction division, grain count over a chromosome set at MII should be half that over MI chromosomes, assuming that ultraviolet penetration and tritium self-absorption is the same in both situations. At a dose of 30 J m^{-2} , corrected grain counts over MI and MII chromosomes were not significantly different ($P \approx 0.2$), whereas at 60 and 120 J m^{-2} , MI grain counts were approximately twice those of MII chromosomes. These results suggest that repair capacities are similar at these two stages, although considerably lower than in the intact GV. Because of the high grain counts over GV-2 stage oocytes, compared with later stages, we determined whether there was a decrease in thymidine uptake during oocyte maturation. Samples of 10 oocytes were incubated in $20 \mu\text{Ci ml}^{-1}$ ^3H -thymidine for 1.5 h, washed in isotonic phosphate buffered saline (PBS) and collected on $5 \mu\text{m}$ Millipore filters by suction filtration. Cells were dissolved in 1% Triton X-100 and samples were counted with a liquid scintillation spectrometer. We found that there was no significant difference in uptake between the GV stage (44.2 c.p.m. per egg, three samples) and the MII stage (52.3 c.p.m. per egg, four samples). Thus, it seems that the higher GV grain counts are due to greater levels of thymidine incorporation into DNA, although higher grain counts could result in part from higher autoradiographic efficiency for the flattened GV nuclei than for condensed chromosomes.

In these experiments mouse oocytes exhibit unscheduled DNA synthesis after ultraviolet irradiation, and thus are capable of excision repair of DNA damage at these stages. Spermatogonia and spermatocytes of several mammalian species also exhibit unscheduled DNA synthesis after ultraviolet irradiation²⁻⁴ or exposure to a chemical mutagen, ethyl methane sulphonate¹⁰. In contrast, spermatids and spermatozoa show little or no unscheduled DNA synthesis with these treatments^{2-4,10}. As the excision repair system seems to act on a variety of base alterations¹¹, it may confer some protection against environmental base damage in mammalian germ cells during meiotic maturation.

The dose rate effects for X-ray-induced mutations of mouse oocytes have been interpreted as indicating the presence of an X-ray repair system during oogenesis^{12,13}. Oocyte radio-

sensitivity follows the same pattern in diverse animals, including mice¹⁴⁻¹⁶. During meiotic maturation, mouse oocytes are the least sensitive to X-ray induction of mutations or chromosome aberrations before GV breakdown, then become more sensitive at MI (ref. 17). Although repair of ultraviolet and X-ray damage to DNA involves several distinct processes, the base-damage component of X-ray damage could probably be repaired during meiotic maturation stages of oocytes, and changes in excision repair capability may account in part for the known changes in X-ray sensitivity. In addition to providing protection from genetic damage at meiotic maturation stages, the excision repair capacity seen here may contribute to the repair capabilities that have been observed in early stages of mouse embryos⁵.

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Duplex microtubule is a new form of tubulin assembly induced by polycations

INTACT microtubules surrounded by a coat of extra material and stabilised by polycations such as Alcian blue, have been seen by Behnke¹ in brain extracts. A similar structure was reported in preparations of microtubules reassembled *in vitro* when DEAE-dextran was added (ref. 2) and the extra material was attributed to DEAE-dextran itself. In this note and the accompanying communication by Behnke³ evidence is presented that the outer component is an additional shell of tubulin subunits probably complexed with polycations. This structure is related to a double ring form of tubulin, which is a putative nucleating centre for microtubule assembly.

Highly purified tubulin prepared from pig brain⁴ was polymerised at 37 °C to form microtubules. Protamine was added at a twofold molar excess over protein, and there was an immediate increase in turbidity. The microtubules, which had the characteristic 40 Å axial periodicity and 250 Å diameter, were surrounded along their length by an outer wall which had an overall diameter of 380 Å as Fig. 1 shows. Apart from some protamine, protein estimation and sodium dodecyl sulphate (SDS) gel electrophoresis show that the protamine-treated tubules consist of tubulin (97%) and the high molecular weight components known to be associated with microtubules². Too little non-protein material is present (< 10%) to account for the outer wall. Since this structure is formed with such a variety of polycations^{2,3} and consists almost entirely of tubulin, it is a new tubulin assembly which we propose to call a duplex microtubule.

When protamine at less than equimolar concentration with respect to protein is added, regions of both duplex and microtubule are seen along any one length of tubule (Fig. 1a). The protofilamentous appearance of the microtubule is also visible at the centre of the duplex, whereas the predominant feature of the outer wall, seen clearly at the edges of the complex, is an axial repeat of 50 Å. Optical diffraction patterns of the duplex structure (Fig. 2) are consistent with these observations. Weak but clear off-meridional reflections corresponding to an axial spacing of 40 Å are seen which are characteristic of patterns from axonemal⁶ and reconstituted⁵ microtubules. Also there are strong off-meridional reflections at $1/50 \text{ Å}^{-1}$ which we have associated with a helical arrangement of subunits in the outer wall. The whole structure contributes to the equatorial pattern and for this reason the other reflection characteristic of microtubules, namely an equatorial at 50 Å (refs 5 and 6) has not been clearly identified. The pattern also shows other reflections; these and the ones we have described indicate that the duplex structure is complex. A simpler structure which resembles the outer wall by itself is, however, obtained by adding spermine to tubulin at 4 °C. (Fig. 1c). Its diameter is similar to that of the duplex tubule and the 50-Å axial periodicity can be seen. Its diffraction pattern (not shown) shows the strong 50-Å off-meridional spots and in addition strong near equatorial reflections at a spacing corresponding to 40 Å. The dimensions of this lattice (50 Å axially $\times$ 40 Å azimuthally) are therefore the same as the size of the subunits in a microtubule (40 $\times$ 50). This implies that the subunits in both the inner and outer wall of the duplex tubule are the same but oriented perpendicular to each other in the two layers of the complex.

The structure of the duplex microtubule was investigated further by comparing its stability with that of microtubules at 4 °C. The ring forms produced on cooling are summarised in Table 1. Microtubules, in the absence of protamine, are completely dissociated after 30 min at 4 °C into single rings of outer diameter 380 Å, inner diameter

250 Å (30–36S protein)^{2,7} and particles, possibly dimers (6S protein)^{2,7}. These single rings have the same outer diameter as the duplex tubules and are approximately the same as the outer diameter of the single rings, double rings and helices reported previously for similar conditions^{2,8}.

In the presence of protamine, duplex and microtubules are seen even after 1 h at 4 °C as well as single rings and double rings (Fig. 1b). Both these rings have the same outside diameter as the duplex microtubules (Table 1) as seen fortuitously in Fig. 1b where a ring is superimposed on a tubule. Double rings together with short segments of duplex

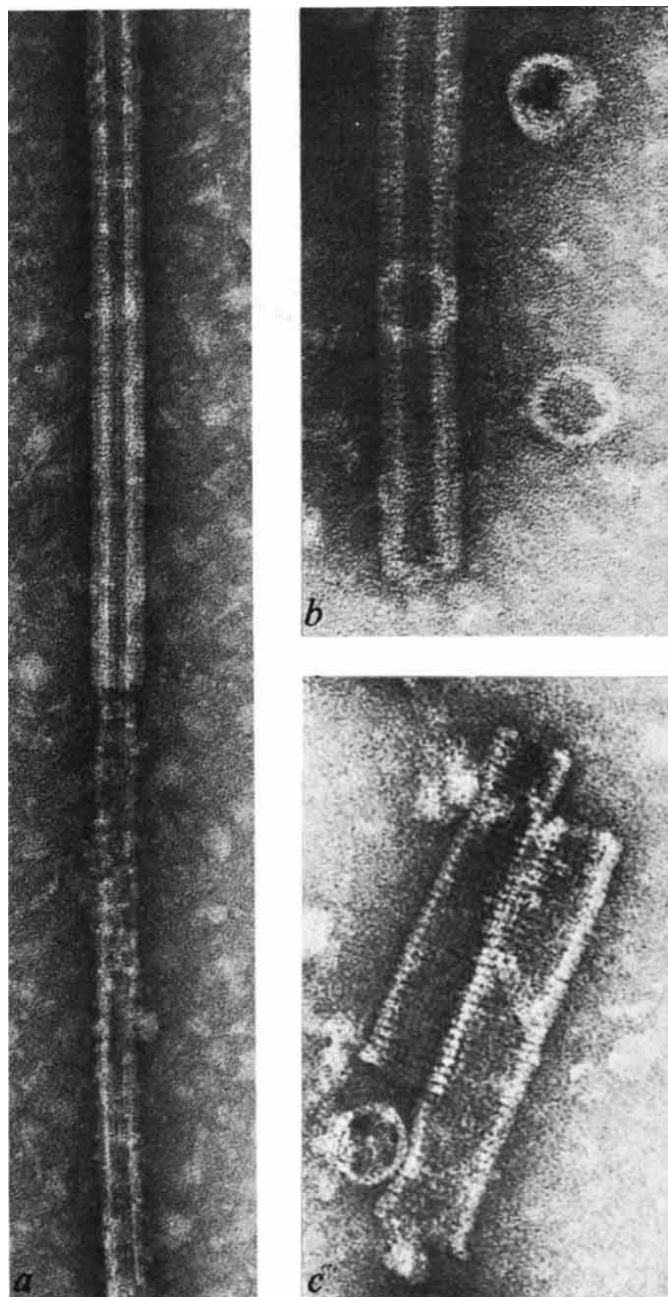


Fig. 1 Polymorphic forms of tubulin. *a*, Regions of duplex microtubule induced by low levels of protamine. Microtubules from purified tubulin⁴ (10 μM) in reassembly buffer (100 mM MES, 1 mM EGTA, 0.5 mM Mg^{2+} , 1 mM GTP, pH 6.8) were incubated with neutralised protamine (5 μM). ($\times 150,000$.) *b*, Duplex tubules cooled at 4 °C for 1 h. The preparation shows both undissociated duplex tubules and ring forms. The fortuitous superposition of the single ring on the duplex tubule illustrates that their diameters are the same. ($\times 250,000$.) *c*, Spermine-induced tubule formation. Purified tubulin (1.8 μM) was incubated at 4 °C with neutralised spermine (1.5 μM), giving rise to a structure resembling the outer wall of the duplex structure. ($\times 250,000$.) Samples for electron microscopy were negatively stained with 2% uranyl acetate.

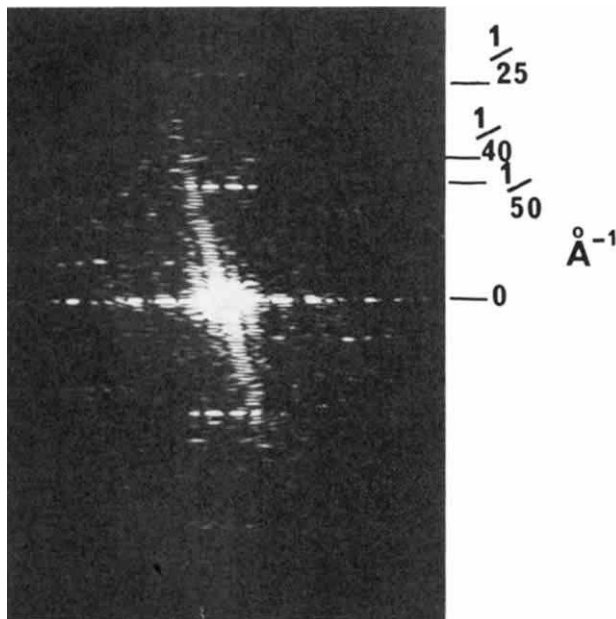


Fig. 2 Optical diffraction pattern of a duplex tubule showing 40-Å microtubule reflections and the 50-Å off-meridional reflections from the outer wall. The 50-Å reflections are also seen in patterns from spermine induced tubules.

again with diameter 380 Å were formed by adding protamine to tubulin at 4 °C, (Fig. 3). These rings are so well preserved that often 13 subunits (the number of protofilaments in a microtubule) can be counted in the inner ring (Fig. 3, inset). Significantly the outer diameter of this inner ring is 250 Å, the same as the microtubule. The double ring therefore has the same cross section as the duplex (Table 1). The subunits in the outer ring cannot be counted, suggesting that the molecules are differently arranged.

Some of the structures induced by polycations may also be formed *in vivo*. The duplex microtubule is seen occasionally in brain extracted with a special microtubule stabilising medium¹. Macrotubules of diameter about 340 Å are formed in cells subjected to cold⁹, high pressure⁹ and drugs¹⁰. One form¹⁰, in particular, is found in the presence of vinblastine, itself a polycation. It is not, however, clear from these observations whether these structures are the same as the duplex tubule or the outer wall, as in the spermine-generated tubules.

The formation of the duplex tubule *in vitro* may provide clues to the mechanism of microtubule assembly which is at present unclear. Previous suggestions have involved the unrolling of rings into protofilaments^{7,8}, or the rearrangement of stacks of rings into a helix², but both pro-

posals require large subunit rearrangement. Another possibility follows from the observations presented here. Before tubule formation the dominant form, at least in our preparations, is the 380-Å diameter single ring (or short segment of helix). A ring form (although not necessarily the same one for each proponent) is generally agreed to be a necessary step in polymerisation and a factor which converts 6S protein to a ring form has been described by Weingarten *et al.*¹².

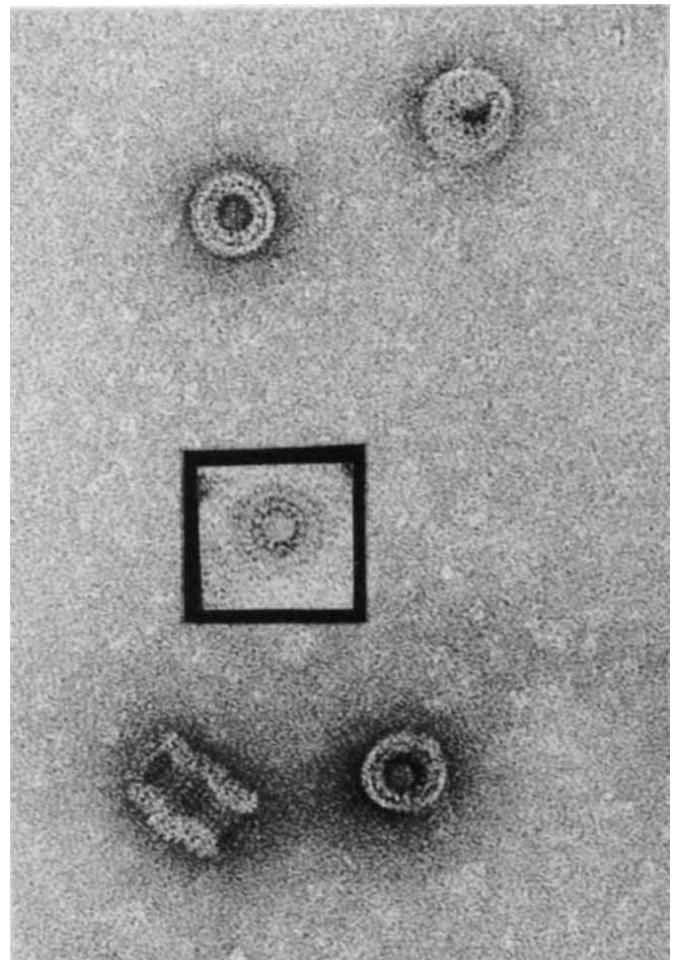


Fig. 3 Double rings and short segments of duplex tubule seen when protamine is added to tubulin at 4 °C. Insert shows double ring with 13 subunits in the inner ring. The samples were negatively stained with 2% uranyl acetate. ($\times 250,000$.)

Table 1 Diameters of the ring forms of tubulin

Type of ring	Conditions	Diameters (Å)*			
		Inner ring		Outer ring	
		Inner	Outer	Inner	Outer
Double ring	Tubulin + protamine at 4 °C	120	250	250	380
Double ring	Tubules + protamine cooled to 4 °C	120	250	250	380
Single rings	Tubules + protamine cooled to 4 °C	—	—	250	380
Single rings	Tubulin 4 °C	—	—	250	380

* Range of diameter measurements $\pm 20\%$.

We consider a plausible sequence of events is first the formation of the 380-Å diameter ring or segment of helix from 6S protein. This structure then serves as a template for the formation of a second ring or segment of helix on the inside. The double ring is stabilised by polycations but could occur naturally in small amounts and is possibly the disk first described by Borisy *et al.*¹¹. Polymerisation proceeds by growth of this structure to give a short segment of duplex tubule. This short segment then acts as a template for microtubule polymerisation, the microtubule growing from the inner ring. In support of this model, it has been shown that 6S tubulin which does not normally polymerise by itself, will assemble onto the inner wall of a short length of duplex². Also double rings induced by low levels of protamine are incorporated into normal microtubules on polymerisation.

Further studies are in progress to test this model and to determine the relationship between the surface lattices of the various tubule forms.

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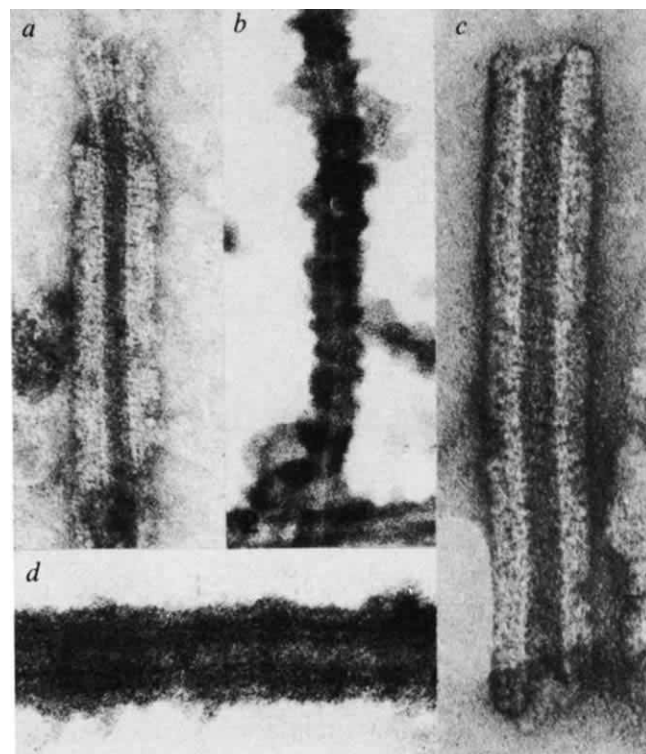


Fig. 1 *a*, Negatively-stained microtubule showing the outer component ($\times 250,000$). A crude preparation of brain microtubules was made in the following way. Cow brains were briefly homogenised in stabilising medium (50% glycerol, 10% dimethyl sulphoxide, 5 mM $MgCl_2$, and 5 mM phosphate buffer, pH 7), at room temperature. The homogenate was centrifuged, first at 12,000*g* for 20 min and then at 40,000*g* for 4 h. The final pellet was resuspended in a small volume of stabilising medium and samples were applied to carbon-coated grids. They were stained with 1% uranyl acetate and examined in the electron microscope. *b*, Longitudinal section of isolated microtubule ($\times 160,000$). The preparation was fixed in a mixture of 2% glutaraldehyde and 4% tannic acid⁵, without post-osmification, and embedded in araldite by standard techniques. *c*, Protamine-induced decoration of microtubule, negatively stained ($\times 300,000$). Crude preparations of brain microtubules prepared as in *a* (ref. 1 and O.B., unpublished), were resuspended in stabilising medium containing 0.1 mg ml⁻¹ protamine sulphate. They were incubated for 4-10 h in this medium before examination in the electron microscope. *d*, Longitudinal section of protamine-treated microtubule ($\times 240,000$). The preparation was fixed in a mixture of 2% glutaraldehyde, and 4% tannic acid as in *b*.

An outer component of microtubules

I HAVE described (ref. 1 and O.B., unpublished) a method by which microtubules from blood platelets or brain may be isolated in an intact form. They are preserved in a medium containing glycerol and dimethyl sulphoxide and can be collected in large amounts by differential centrifugation. This procedure differs from the usual methods of isolation which begin with unpolymerised tubulin, the protein of which microtubules are comprised^{2,3}, and has the advantage that structures associated with them in the cell are retained (O.B., unpublished). In the electron microscope these appear as a coating around the microtubule wall which is usually of an ill-defined amorphous nature (Fig. 1*b*).

Occasionally the microtubules show a distinctive structure of regular morphology (Fig. 1*a*). This is seen in negatively-stained preparations as a thickening of the microtubule wall, of limited extent longitudinally, in which the outer diameter is increased to 360-390 Å, but the inner lumen remains at 120-130 Å (Fig. 1*a*). The outer component often appears as stacked disks with a periodicity of 50 Å and inclined at an angle of 75° to the long axis, but sometimes a looser structure seems to be wound around the microtubule.

I found that a very similar kind of outer component could be induced at will. Treatment with various compounds caused the microtubules to aggregate and on examination in the electron microscope most of them were seen to have thickened walls. The compounds which did this were Alcian blue 8GX (1%), protamine sulphate (0.1 mg ml⁻¹), polylysines (molecular weights 1,000-30,000; 0.1 mg ml⁻¹) and DEAE-dextran (0.4 mg ml⁻¹), (ref. 4), all of which carry multiple positive charges at the pH of the stabilising medium. The outer components they produced were closely similar to those described above and were made of distinct globular subunits about 50 Å in diameter (Fig. 1*c*). In specimens fixed in glutaraldehyde and tannic acid⁵ and sectioned, the two layers of the wall were clearly visible (Fig. 1*d*).

Thus some isolated brain microtubules are decorated with a "natural" outer component but decoration can be induced on virtually all microtubules in a preparation by incubation with certain cationic compounds. There is no proof that the natural and the induced outer components are the same, but their ultrastructural appearance is very similar.

The outer component may also be formed on purified microtubules. Preparations which have been depolymerised, and polymerised in stabilising medium many times, and which are pure by SDS-gel electrophoresis, still give extensive decoration on treatment with cationic compounds. Similar observations have been made⁶ with microtubules that have been purified in a different way. Because the outer component seems to comprise a major part of the decorated tubules it seems reasonable to suppose that it can only be made of tubulin.

If the extra layer is composed of tubulin then it must be bound in a specific fashion. The subunits of the outer component are regularly placed and prolonged incubation with cationic compounds for up to 1 week does not result in the deposition of additional layers. Also, the sheets of tubulin which may be formed in some conditions⁶ become decorated only on one side, implying that only the outer side of the microtubule wall may be covered in this way.

The ease with which the outer component can be induced, and the fact that it is occasionally seen in freshly isolated microtubules, suggest that it could be present in the living cell. Structures of this precise nature have not been seen

in thin sections of cytoplasm but it may be relevant that microtubules often seem to be surrounded by a halo of low density⁷. This "clear space" from which other cytoplasmic constituents are excluded, has about the same width as the outer component, and it is not impossible that the two are in some way related.

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Foetal erythropoiesis in human leukaemia

DURING human development changes occur in the red-cell proteins and antigens¹. At birth the predominant haemoglobin (Hb) is Hb F with approximately 20% and less than 1% of the major and minor adult haemoglobin components (Hbs A and A₂) respectively. Hb F at birth is a mixture of two molecular species, one with glycine at position 136 of the γ chain ($\alpha_2\gamma_2^{136(\text{Gly})}$) and the other with alanine at this position ($\alpha_2\gamma_2^{136(\text{Ala})}$). The γ chains are designated $^G\gamma$ and $^A\gamma$ respectively and at birth the ratio of $^G\gamma/^A\gamma$ is approximately 3:1 (ref. 2). Foetal red cells contain low levels of carbonic anhydrase isozymes B and C and react strongly with anti-i antibody but weakly with anti-I. During the first 12 months the level of Hb F falls and it is replaced by Hbs A and A₂. The ratio of $^G\gamma/^A\gamma$ chains changes to 2:3 in the small amount of Hb F found in adult life³. The red cells lose their reactivity to i antibody and increase their reactivity to I. The carbonic anhydrase isozymes increase to adult levels by the end of the third year⁴. These changes act as markers for the change from foetal to adult erythropoiesis.

There are several examples of the emergence of foetal proteins during neoplastic transformation⁵. Since at least some human leukaemias involve red-cell as well as white-

cell precursors there may be a reversion to a foetal form of erythropoiesis in these disorders. The only condition where there is a marked increase in Hb F production is juvenile chronic myeloid leukaemia (JCML)⁶⁻¹⁰. We described⁷ a child with this disorder in whom there was a gradual increase in the Hb F level and a reduction in the amounts of Hbs A and A₂ and the carbonic anhydrase isozymes B and C over a period of 12 months. Thus as the disease progressed there was a gradual reversion to foetal erythropoiesis. In the study reported here we have confirmed this observation and shown that foetal erythropoiesis occurs occasionally in other forms of leukaemia.

Our subjects were nine infants or young children with JCML; 64 children and adults with acute myeloblastic leukaemia; 10 with erythroleukaemia; 19 with acute lymphoblastic leukaemia; 30 with chronic myeloid leukaemia and 44 with chronic lymphatic leukaemia. Haematological studies, haemoglobin and carbonic anhydrase isozyme analysis by starch gel electrophoresis, and Hb F and A₂ estimations followed previously described methods^{11,12}. The intracellular distribution of Hb F was assessed by acid elution¹³ or fluorescent anti-Hb F-antibody techniques¹⁴. The relative rates of α -, β - and γ -chain production were estimated by ³H-leucine incorporation¹⁵ and the glycine composition of peptides γ 15 or CB3 was determined as described previously^{2,16,18}.

The results of haemoglobin and red-cell enzyme analysis on the erythrocytes of 10 infants or children, nine with JCML and one with erythroleukaemia, are summarised in Table 1. The ages ranged from 3 months to 5 yr and the clinical and haematological findings were as in previously reported cases of JCML^{6,7}. The Philadelphia chromosome was absent in each case. In all but two cases high levels of Hb F in the 40-80% range were found, and these increased during the evolution of the illness. The Hb F was heterogeneously distributed among the red cells. In two cases where the fluorescent anti-Hb F-antibody technique was used the fluorescent cells constituted 60.3 and 100% of the total whereas the levels of alkali resistant haemoglobin were 30.2 and 69.5% respectively, indicating that a proportion of cells must have contained both Hbs A and F. In the cases with high levels of Hb F the glycine composition of peptides γ 15 or γ CB3 ranged from 0.69 to 0.81; that is, in the range found in normal cord blood samples². In one case (case 8, Table 1) sequential estimations of the relative rates of α -, β - and γ -chain synthesis were obtained (Fig. 1). Initially γ chains made up approximately 45% of the total non- α -chains produced, which correlated well with the initial Hb F level of 38%. In a sample obtained 4 weeks

Table 1 Haematological data and haemoglobin and red cell enzyme analysis on 10 infants or young children with JCML or erythroleukaemia (case 8)

Case no.	Age (yr)	Sex	Days from presentation	Hb (g dl ⁻¹)	Platelets ($\times 10^6$ l ⁻¹)	WCC ($\times 10^6$ l ⁻¹)	Hb F (%)	Hb A ₂ (%)	Glycine residues in γ 15 or CB3	Carbonic anhydrase B and C
1	2	F	0	9.2	80,000	48,000	20.0	1.6	—	Reduced
			250	10.3	<10,000	19,600	65.5	<0.2	0.84	Absent
2	2	M	100	8.6	<10,000	53,300	48.9	0.7	—	Absent
3	3 months	M	20	9.1	77,000	68,000	45.5	1.0	0.79	Reduced
4	5	M	10	10.2	15,000	15,700	50.0	<1.0	0.81	Absent
5	3	M	5	8.2	8,000	42,000	38.2	0.98	0.69	Reduced
6	4	M	0	11.0	32,000	68,000	70.0	<1.0	—	—
			60	10.6	36,000	7,200	69.9	Absent	0.83	Absent
7	5	M	0	11.0	38,000	15,700	30.2	<0.02	0.75	Absent
			100	12.5	31,000	17,900	32.8*	<0.2	—	Absent
8	3½ months	M	0	7.4	<10,000	10,000	42.7	1.2	0.78	Absent
			28	7.9	<10,000	1,800	85.0†	—	—	—
9	3 months	M	0	10.2	52,500	30,800	7.2	2.7	0.81	Normal
			450	9.6	20,000	36,800	1.9	3.1	—	—
			550	7.7	10,000	308,000	13.0	2.0	—	Normal
10	3 months	M	1	9.2	105,000	45,600	2.0	—	—	—
			100	11.0	200,000	35,000	4.9	1.4	0.56	Normal

* This child had 50% Hb F production by ³H-leucine incorporation studies at the time that the peripheral blood showed 32.8% Hb F.

† Obtained from ³H-leucine incorporation data.

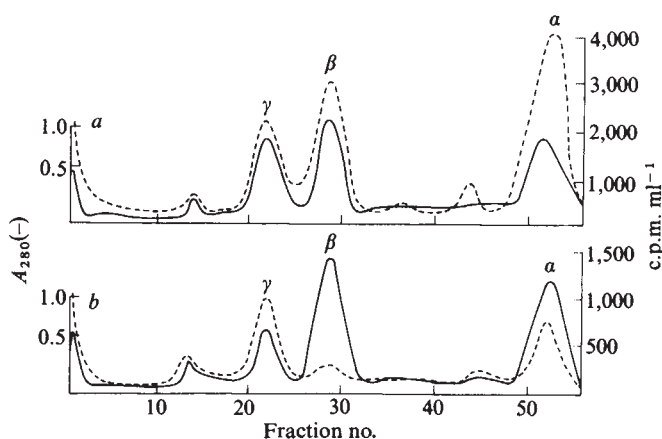


Fig. 1 Elution profiles of globin chains separated by CM-cellulose chromatography in a 8 M urea-mercaptoethanol-phosphate buffer system, pH 6.7. Peripheral reticulocytes and nucleated red cells were incubated for 1 h with ^3H -leucine in the conditions indicated in the text. Cells were then washed, lysed and total cell globin was prepared by the acid-acetone method. The globin chains were separated and the radioactivity incorporated into each chain was determined. Profile *a* is taken from a child with erythroleukaemia just after presentation. Profile *b* was prepared from the cells of the same child 28 d later. There is more β chain carrier in *b* because the child was receiving blood transfusions but it is clear there has been almost a total reversion to γ chain synthesis during the evolution of the illness.

later, γ chains constituted about 80% of non- α -chains produced, indicating that shortly before he succumbed he was producing more than 80% Hb F.

The highest levels of Hb F in the infants and children with JCML and erythroleukaemia were associated with a reduction in the levels of Hb A₂ and carbonic anhydrase B and C (Table 1) and in several cases none was detectable. In these cases the starch gel electrophoretic patterns of the red cell lysates were indistinguishable from those of normal newborn infants. Embryonic haemoglobin was not present. In these cases there was an elevation in the i antigen titre but only in one was there a significant fall in the level of I. Case 6 showed in addition to 70% Hb F, about 3% Hb Barts, but family studies indicated that in addition to having JCML he had received an α -thalassaemia gene from his mother. No haematological abnormalities were found in any other family members and the identical twin in case 3 was completely normal.

Cases 9 and 10 did not follow the pattern outlined above. Case 9 survived much longer, had a higher white cell count and his Hb F level rose to only 13%. Case 10, while having a clinical presentation identical in every way to JCML, ran a milder course and did not produce more than 4% Hb F. Peptide $\gamma 15$ prepared from the latter had a glycine composition of 0.56 residues which is in the range found in the small amounts of Hb F present in normal adults³.

Elevated levels of Hb F were observed also in 43 out of 64 patients with acute myeloblastic leukaemia, 7 out of 8 patients with erythroleukaemia, 12 out of 19 with acute lymphoblastic leukaemia, 19 out of 30 with chronic myeloid leukaemia and 15 out of 44 with chronic lymphatic leukaemia. In most cases the level did not exceed 5% of the total haemoglobin and only in three cases were values in the 10–25% range encountered. No consistent alterations in the levels of Hb A₂, carbonic anhydrase isozymes or I antigens were found. The glycine composition of $\gamma 15$ of the Hb F varied widely, as has been noted by others¹⁰. Hb F levels were followed in a group of 17 patients with acute myeloblastic leukaemia for periods ranging from 1 to 15 months. Fifteen of the seventeen developed an increase in the level of Hb F about 60 d after the commencement of treatment at a time when, following bone marrow aplasia due to

chemotherapy, rapid regeneration was taking place. Significantly greater increases of Hb F levels were observed in those achieving a remission. Details of these patients will be reported elsewhere.

The results indicate that in some forms of leukaemia in early childhood there may be an almost total reversion to a foetal form of erythropoiesis. Levels of Hb F may reach those of cord blood. The levels of Hb A₂ and carbonic anhydrases B and C fall to produce an electrophoretic pattern of red-cell proteins identical to that observed in newborns. This pattern of erythropoiesis, found most commonly in JCML, can occur also in erythroleukaemia and it does not occur in every case of JCML. This may, of course, simply reflect the heterogeneity of JCML²⁰. It was not seen in other forms of leukaemia and the slight elevation of Hb F in these disorders is associated in many cases with recovery from bone marrow aplasia caused by chemotherapy. When the reversion to foetal erythropoiesis occurs it provides a marker for the presence of the neoplastic cell line.

Why does erythropoiesis revert to a foetal form during the evolution of some types of leukaemia in early childhood? It is possible that it is another example of the reversion to foetal protein synthesis which occurs with other neoplasms⁵. If this is the case, however, there is no reason why it should occur much more frequently in infancy. Its occurrence at this time suggests that it is related to the normal mechanism of differentiation of red-cell proteins during maturation. Although by the end of the first year most erythrocytes contain mainly Hb A, cell lines persist which produce relatively large quantities of Hb F^{14,21}. Such 'F cells' are produced in adults in relatively constant numbers, suggesting that some stem-cell lines do not undergo the normal switchover from Hb F to Hb A production. It has been suggested that this switch is hormonally controlled^{1,22} and that the 'F cell' population in adults has arisen by chance in stem cells which did not come under its influence. Thus the chances of a leukaemic transformation occurring in a line making predominantly Hb F would be much greater in infancy and therefore the reversion to foetal erythropoiesis should be much commoner at this age. Similarly the chances of it occurring in adults would be extremely small although an occasional case might occur²³. Thus the findings are fully consistent with the idea⁷ that these leukaemias arise in cell lines which have escaped the normal processes of differentiation during development.

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Evidence for Hb Lepore-like hybrid globin β chain genes in mice

WE have described structural data for mouse and rabbit embryonic β -like ϵ chains and the conclusions which can be drawn with respect to the evolution of ϵ globin chain genes in both species¹. Here we present evidence for crossover events in the ancestral mouse β and ϵ -type chain genes leading to Hb Lepore-like globin chain genes which are functional in mice of recent evolutionary origin.

BALB/c mice, which we used for this study, have two types of embryonic β -like ϵ chains— $\epsilon\gamma$ and $\epsilon\zeta$ (refs 2–7). Based on the composition of tryptic peptides of the $\epsilon\gamma$ and $\epsilon\zeta$ chains,

the tentative sequence of both chains was established using the adult mouse β chains as a reference^{2,5,8,9}. BALB/c mice have two linked genes for the adult β chains, which control the synthesis of the β major and minor globin chains, respectively. The two chains differ at six positions (Nos 9, 16, 20, 58, 72 and 76)⁸.

$\epsilon\gamma$ and β minor chains are closely related since they are identical at four of the six positions at which β major and minor are different. The other two positions are different in $\epsilon\gamma$ from both β major and β minor^{1,2}. Therefore the β minor chain was used for comparison of the structure of the ϵ and β chains. $\epsilon\zeta$ and $\epsilon\gamma$ chains are unrelated apart from their β -like character¹. A comparison of the $\epsilon\gamma$ and β minor chains with respect to the sites of amino acid substitutions shows that most substitutions are located at the amino and also some at the carboxy-terminal ends of the $\epsilon\gamma$ chain, whereas the central part of the chain is almost identical to the β minor chain (Fig. 1).

Rather the reverse is true for the $\epsilon\zeta$ and β minor chains. Here the substitutions are concentrated at the centre of the $\epsilon\zeta$ chain whereas both the amino and carboxy terminal ends look very similar to the β minor chain. Since some peptide composition data of both ends of the ζ chain are, however, still missing, the situation is not as clear as for the $\epsilon\gamma$ chain. The presence of an amino terminal peptide though, which is identical to the adult β -chain peptide composition, leads us to predict close resemblance of the amino terminal part of the $\epsilon\zeta$ chain and of the β minor chain (Fig. 1).

The reciprocal nature of the two ϵ chains as compared with the adult β chains as shown in Fig. 1, suggests that either the β -chain genes or the ϵ -chain genes arose by a crossover event between non-identical genes. Either a single or a double crossover could explain our data. The double crossover interpreta-

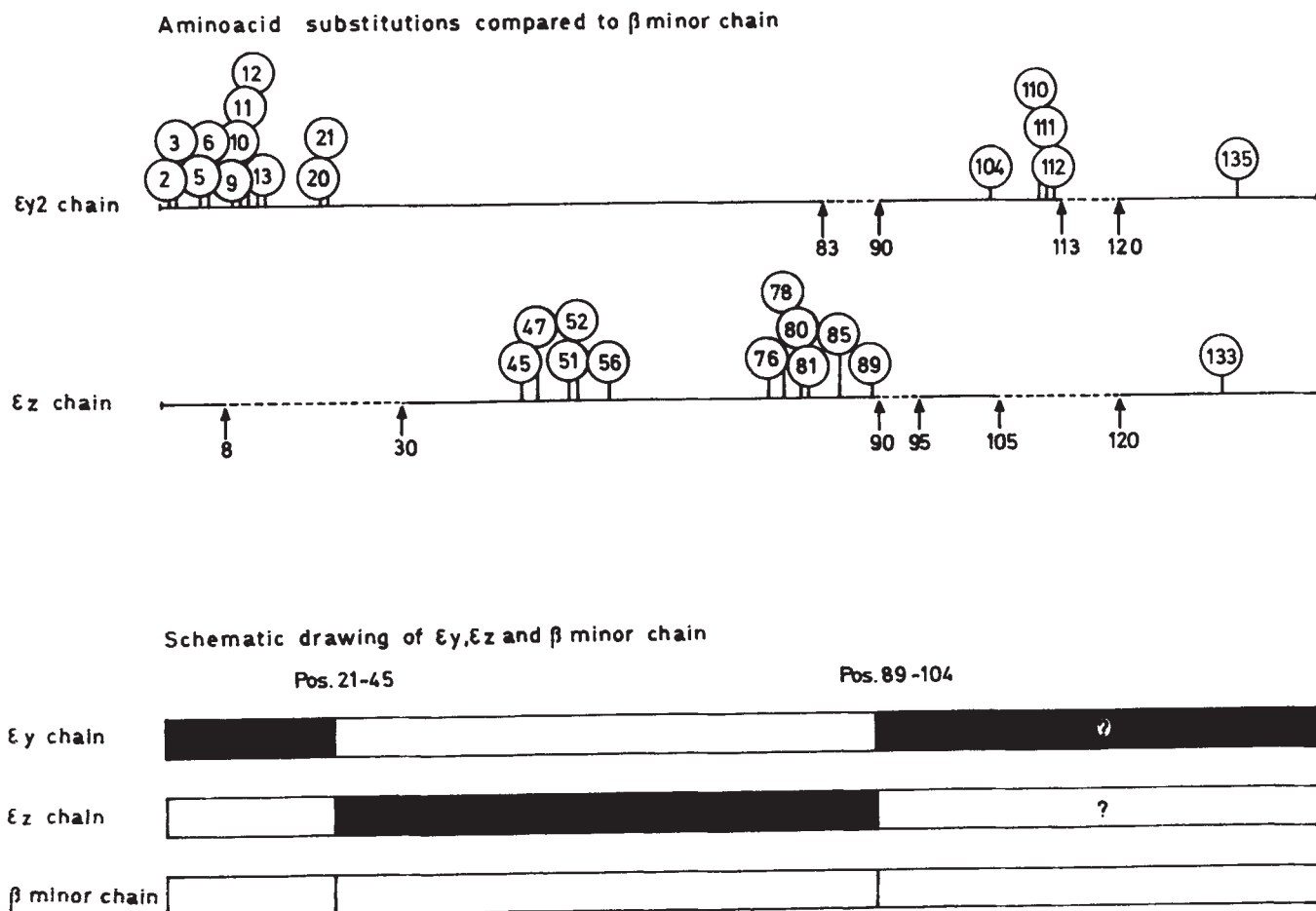


Fig. 1 Unequal distribution of amino acid substitutions is found in the embryonic $\epsilon\gamma$ and $\epsilon\zeta$ chains of the mouse as compared with the adult β minor chain. This suggests an origin of either adult β -type or embryonic ϵ type chains by crossover mechanisms, as indicated by the schematic drawing. The most likely crossover models are depicted in Fig. 2.

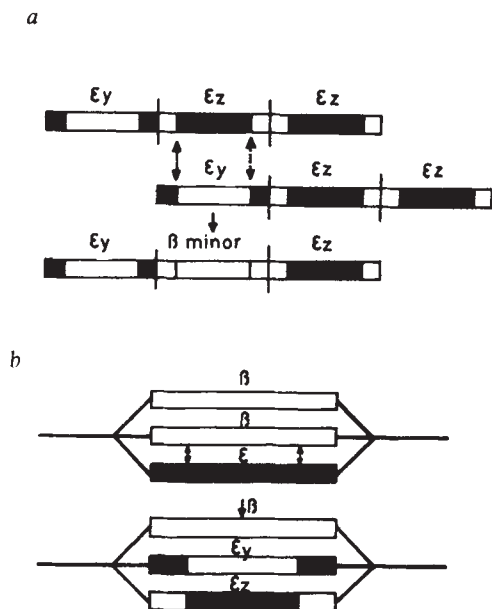


Fig. 2 Crossover models which may account for the presence of Hb Lepore-like hybrid globin β or ϵ -chain genes in mice. Two theoretical possibilities can be assumed for the distribution of amino acid substitutions as shown in Fig. 1. (1) Two ancestral genes, that is the ancestral ϵy and ϵz -chain gene form one new gene, the β minor chain gene. (2) Two ancestral genes, that is the ancestral ϵ and β -chain gene undergo a crossover event thus creating two new genes, the ϵy and ϵz -chain genes. In principle two models can account for the observed phenomenon of Hb Lepore-like hybrid globin chains. First, the model of misalignment and unequal crossover of closely related genes, which is normally used for the explanation of Lepore haemoglobins. Second, a crossover mechanism involving identical and non-identical genes arranged in parallel on one chromosome. A model of parallel arranged DNA double strands located within one chromosome has for example been proposed by Smithies for the genes of immunoglobulin chains¹¹. The first crossover model of misalignment and unequal crossover only applies to hypothesis (1), that is, two ancestral genes form one new gene at least under the premise of one temporal event. Otherwise one has to assume additional processes of genetic segregation and recombination first separating and then bringing together again the two newly formed genes. The second crossover model involving genes arranged in parallel would account for hypothesis (2), namely two ancestral genes create two new genes, especially under the additional assumption of one single temporal step.

tion would have to be supported by more structural data, mainly on the ϵz chain.

Two main mechanisms are considered here to interpret the crossover origin of mouse β or ϵ -chain genes. The first mechanism would involve unequal Lepore-type crossing over between related non-allelic genes¹⁰, the second would involve reciprocal crossing over between allelic non-identical but closely related genes arranged in parallel on the same chromosome¹¹ (Fig. 2).

We made use of minimal assumptions for explanation of the data. A single temporal event using the generally accepted colinearity of all genes would be the easiest interpretation. If one accepts one single meiotic crossover event to account for the structure of all three β -like genes, ϵy , ϵz and β minor, then the most likely origin would be explained by the Lepore hypothesis (Fig. 2). We assume that the ϵy -chain gene is the ancestral ϵ -chain gene, since ϵy is the only ϵ chain which combines with the embryonic α -type chain χ to form haemoglobin E₁. The ϵz chain apparently only combines with the adult α chain^{2,3}. Then the ϵz chain might have been the ancestral adult β -type gene. An unequal crossing over (Fig. 2) between these two genes could have generated the adult β gene (anti-Lepore) which was closer to β minor than to β major. A further independent duplication of β minor would then have been the basis for the evolution of an independent β major gene.

This Lepore hypothesis has to be modified if a double crossover would have to be assumed to account for the reciprocal nature of three pieces of the ϵy and ϵz -chains as compared with the β chain (Fig. 1). A prerequisite for this model would be that the ϵz -chain gene was duplicated before the double crossover event. After the double crossover, the β -chain gene would then be between the two ϵ -type genes. This would naturally have consequences for the regulation of coordinated expression of both ϵ -chain genes.

The hypothesis of parallel not colinear genes, if extended to the β -globin loci, would not present any difficulties in the creation of the ϵy , ϵz and β -chain genes, either in meiotic or in other germ line cells. No difficulty would arise for single or double crossovers. No loss or gain of genetic material would occur (Fig. 2). In this model, the β -chain genes do not need to be the result of a crossover between ϵy and ϵz -chain genes. A reciprocal crossover between an ancestral β and ϵ -chain gene could have formed the ϵy and ϵz -chain genes (Fig. 2). The hypothesis just described and the hypothesis of unequal crossover for the origin of Lepore-type globin chains can be checked in patients with Lepore haemoglobin, especially in the homozygous state. The data for one homozygous individual with Hb Lepore Boston¹⁰, who shows no detectable amounts of anti-Lepore globin seem to favour the unequal crossover model.

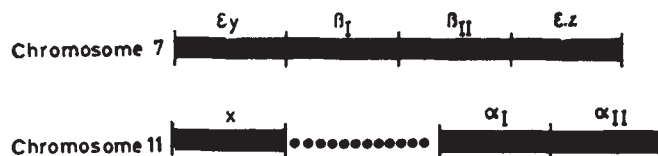


Fig. 3 Hypothetical arrangement of α and β -type chain genes of BALB/cJ mice on chromosome 11 and 7, respectively. The location of adult α and β -chain genes on their respective chromosomes is known, as is the tight coupling of ϵy and adult β -chain genes. From our crossover hypothesis involving ϵy , ϵz and adult β -chain genes, a coupling of all β and ϵ -chain genes seems to be likely as well as the location of adult β -chain genes between embryonic β chain genes. The information on location and coupling of α -type chain genes was included for the sake of completeness¹⁵. The assumption that the χ chain gene is linked to the adult α globin chain genes is based on structural homology to the adult α chains and also on functional aspects such as mutual replacement of χ and α chains during ontogenesis¹⁶.

Lepore-type β chain has been reported in a species of mouse (*Mus musculus caroli*)¹². Therefore it seems possible that crossover events have been important in the evolution of murine β -chain genes.

In rabbits as in mice, evidence has been obtained for multiple embryonic β -type ϵ chains¹. These chains look much more similar to each other and the distribution of amino acid substitutions compared with the adult β chains of rabbits seems more even than in mice. Therefore we assume that no crossover was involved in the generation of the β -type genes in the rabbit.

The likelihood that either the existing β -chain genes of BALB/c mice or possibly both ϵ -chain genes arose by recombination events suggests that both ϵ -chain genes are closely linked with the β -chain loci (Fig. 3). Linkage of the β minor and β major chain genes^{12,13} and of both to ϵy ^{4,7} on chromosome 7¹⁴ has been shown before.

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Embryonic ϵ chains of mice and rabbits

THE existence of an embryonic α -type globin chain (χ or ζ) in early embryos of mice and rabbits has been described¹⁻³. A similar ζ chain can be found in humans^{4,5}. Here we present data on the structure of the embryonic β -type ϵ chains of mice and rabbits. In mice, three embryonic haemoglobins, Hb EI, EII and EIII have been described. These haemoglobins consist of four globin chains, namely the embryonic χ , $\epsilon\gamma$ and $\epsilon\epsilon$ chains and the adult α chain^{3,6-8}. Hb EI has the structure $\chi_2 \epsilon\gamma_2$, Hb EII is $\alpha_2 \epsilon\gamma_2$ and Hb EIII is $\alpha_2 \epsilon\epsilon_2$. The χ chain is an embryonic α -type chain^{1,2}. We find six haemoglobin fractions in nucleated embryonic erythrocytes of the rabbit. Three of the haemoglobins (Hb EI-EIII) do not contain α , but χ chains like Hb EI of the mouse; the other three (Hb LI-III) are similar to mouse Hb EII and EIII¹.

All non- α -type embryonic globin chains of both species are β -type ϵ chains. We find less structural similarity between the ϵ chains of different species than between the β and ϵ chains of the same species. This indicates, in contrast to the embryonic χ chains, that ϵ chains diverged by independent evolution from the ancestral β chains of mice and rabbits.

The ϵ chains of 12-13-d-old embryos of BALB/c mice and of 14-d-old embryonic New Zealand white rabbits were digested by trypsin, and the tryptic peptides were separated by the fingerprinting method. The amino acid composition of the tryptic peptides is shown in Table 1. They were compared with the β chain sequences of mouse (β major and β minor)^{9,10}

and rabbit¹¹⁻¹³. According to the principle of least dissimilarity, the best correspondence was obtained using the β chains of each species as a reference (Fig. 1). The $\epsilon\gamma$ chain which has erroneously been called $\epsilon\epsilon$ chain previously¹, shows a closer overall homology to the β minor than to the β major chain. In addition, in 4 out of 6 positions, that is in positions 16, 58, 72 and 76, in which the β major and minor chains are different, the $\epsilon\gamma$ and β minor chains are identical. The other two positions are different from both β major and minor chains. For the $\epsilon\epsilon$ chain, similar conclusions concerning the relationship to either of the two adult β chains are not yet possible because of a lack of data.

In the case of the rabbit, different chromatographic fractions of ϵ chain material have been analysed separately by the fingerprint method. The fractions were, however, not resolved sufficiently for conclusions to be drawn as to how many rabbit ϵ chains are present. All the data were therefore pooled (Table 1, Fig. 1). These peptide compositions were compared with the adult globin chains of rabbits. The closest homology was found using the rabbit β chain as a reference as shown in Fig. 1. The homologised sequence shows close similarity to the adult β chain. At several positions, indicated by brackets, two or three different amino acids had to be placed either because peptides with very similar compositions were found at different spots of the fingerprint or because one peptide showed non-integral values for two different amino acids. This proves the presence of more than one rabbit ϵ chain. The degree of homology of rabbit ϵ and rabbit β is similar to mouse ϵ and β chains.

To get an impression whether a common evolutionary origin of rabbit and mouse chains could be supported by our data, we compared the individual peptide compositions of mouse and rabbit β chains⁹⁻¹³ with the corresponding peptides of the ϵ chains (Table 1, Fig. 1). For comparison, we used the mouse β minor chain as reference chain because of close structural relationship to one murine embryonic ϵ chain, namely the $\epsilon\gamma$ chain. In the case of the rabbit, the β chain sequence, as reported by Braunitzer *et al.*¹¹ was taken as reference because it looked somewhat closer to the embryonic ϵ chains of rabbits (positions 52 and 56, Fig. 1) than the allelic sequence^{12,13}. Since we used peptide compositions for comparison, even in the case of established sequences, minimal

Amino acid/ Pos. no.	1	24	73	96	
β rabbit	Val His Leu Ser Ser Glu Lys/Ser Ala Val Thr Ala Leu Trp Gly Lys/Val Asn Val Glu Glu Val Gly		Glu Gly Leu ^{Asn} _{Ser} His Leu Asp Asn Leu Lys/Gly Thr Phe Ala Lys/Leu Ser Glu Leu His Cys Asp Lys/Leu		
ϵ rabbit	(Gly Ser) Met Asx ^[Leu] _[Ile] Pro Asx ^[Ser] _[Ala] Phe Asx Gly ^[Thr] _[Ile]		Gly Phe Lys/Lys ^[Val] _[Met] Ala		
ϵ mouse	His Leu Asx Ala		His Asx Ser Glx Ala Val Phe		
γ mouse	Asx Phe Ala Glx Thr Leu Ile Asx Gly Ala Val Glx		Lys/ ? Leu Asx Leu		
β mouse	Val His Leu Thr Asp Ala Glu Lys/Ser Ala Val Ser Cys Leu Trp Ala Lys/Val Asn ^{Pro} _{Ser} Asp Glu Val Gly		Ser Gly Leu Lys/Asn Leu Asp Asn Leu Lys/Gly Thr Phe Ala Ser Leu Ser Glu Leu His Cys Asp Lys/Leu		
Amino acid/ Pos. no.	25	48	97	120	
β rabbit	Gly Glu Ala Leu Gly Arg/Leu Leu Val Tyr Pro Trp Thr Gln Arg/Phe Phe Glu Ser Phe Gly Asp Leu		His Val Asp Pro Glu Asn Phe Arg/Leu Leu Gly Asn Val Leu Val ^{Val} _{Ile} Val Leu Ser His His Phe Gly Lys/		
ϵ rabbit		Thr Pro? Ala Lys/ ^[Gly Ala Phe] _[Glx Ser Ile] ^[Asx Val] _[Glx Leu]	Lys/ Ala Ala Thr Cys Asx Glx Arg/		
ϵ mouse		Leu Glx	Arg/		
γ mouse		Phe Asx	Lys/ Thr Ser Lys/		
β mouse	Gly Glu Ala Leu Gly Arg/Leu Leu Val Tyr Pro Trp Thr Gln Arg/Tyr Phe Asp Ser Phe Gly Asp Leu		His Val Asp Pro Glu Asn Phe Arg/Leu Leu Gly Asn Met Ile Val Ile Val Leu Gly His His Leu Gly Lys/		
Amino acid/ Pos. no.	49	72	121	144	146
β rabbit	Ser Ser Ala His ^{Asn} _{Asn} Ala Val Met ^{Ser} _{Asn} Pro Lys/Val Lys/Ala His Gly Lys/Lys/Val Leu Ala Ala Phe Ser		Glu Phe Thr Pro Gln Val Gln Ala Tyr Gln Lys/Val Val Ala Gly Val Ala Asn Ala Leu Ala His Lys/Tyr His		
ϵ rabbit	^[Ile] _[Ser] ^[Asx Ala] _[Asx Thr] ^[Lys/Gly] _[Met Asx] Tyr ^[Glx] _[Asx Ser]		^[Ala] _[Ser Ser] ^[Leu] _[Ile] ^[Ala] _[Val] ^[Thr] _[Thr]		
ϵ mouse	Leu Thr Val Ala	Glx	Ala Ala		
γ mouse	Ala Ser Gly Pro	Glx	Val Ser		
β mouse	Ser Ser Ala Ser Ala Ile Met Gly Asn ^{Pro} _{Asn} Lys/Val Lys/Ala His Gly Lys/Lys/Val Ile Thr Ala Phe Glu Asp		Asp Phe Thr Pro Ala Ala Glu Ala Ala Phe Gln Lys/Val Val Ala Gly Val Ala Thr Ala Leu Ala His Lys/Tyr His		

Fig. 1 Sequence homology of mouse and rabbit ϵ chains as compared with the corresponding adult β chains. The $\epsilon\gamma$ chain peptide in position 91-95 was taken from Gilman¹⁴. Gilman's sequence data in addition to our peptide data were used for positions 1-30 of the $\epsilon\gamma$ chain¹⁴. In the case of the rabbit, two or three different amino acids had to be placed at the same positions as indicated by brackets. This proves the presence of more than one rabbit ϵ chain. Serine and glycine have been found in addition in the rabbit ϵ chain peptide homologised to the adult β T1 peptide. It seems possible that both residues are linked to the N terminal end of the rabbit ϵ chain(s), as has been found for a minor fraction of adult rabbit α chain, for the embryonic rabbit α chain²⁰ and for the embryonic mouse α T1 peptide (our observations).

differences were determined for all chains. For the calculation we omitted those peptides for which the homology was doubtful. We therefore used for comparison the following homologised stretches: positions 41-82, 96-104 and 121-146 (Fig. 1). In the case of the rabbit for positions 41-59, only the corresponding well fitting peptide was included in the comparison

(Table 1). The $\epsilon\gamma_2$ chain of BALB/c mice has probably asparagine or some unknown residue at position 77, whereas the $\epsilon\gamma_1$ chain may have either lysine or arginine¹⁴. From our data, the presence of asparagine can be excluded (Fig. 1). Of a stretch of peptides totalling 77 amino acids we find the closest homology between mouse β and $\epsilon\gamma$ (3/77 difference) β and $\epsilon\gamma$

Table 1 Amino acid composition of the tryptic peptides of mouse and rabbit ϵ chains

a Rabbit: Residue no. which homologised to known β chain sequences

Table 1. Residue no. which homologues to known protein sequences																																																
Amino acid	1-8		9-17		18-30		31-55		56-59		41-59		60-61		62-65		66-77		67-77		78-82		78-82		83-87		88-95		96-104		105-120		121-132		121-132		121-132		A ^a 133-144		B ^b 133-144		145-146					
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b						
Lysine	0.9	1	1.0	1			2.0	2	1.0	1	1.1	1	1.1	1	1.0	1	1.0	1	1.9	2	0.9	1	0.8	1	1.0	1	1.2	1	1.0	1	2.2	2			0.8	1	1.0	1	0.8	1	1.0	1	1.0	1				
Histidine																1.0	1											1.0	1	1.0	1												1.0	1	1.0	1		
Arginine					0.7	1																							1.0	1	1.0	1											1.0	1	1.0	1		
Aspartic Acid	1.6	2	1.0	1	1.5	1	2.0	2	1.1	1	2.9	3									2.3	2	2.3	2			2.2	1	1.8	2	1.9	2	0.4	0	0.8	1	0 ^a	0					1.0	1	1.0	1		
Threonine			1.0	1	0.6	0.5	2.0	2			0.5	1																1.0	1	1.3	1	1.0	1	1.1	1							1.0	1	1.0	1			
Serine	2.8	3	0.5	0.5			1.0	1			3.5	3																1.0	1		1.2	1	0.5	0	1.0	1	2.0	2							2.8	3		
Glutamic Acid					3.1	3					2.0	2																1.0	1	1.0	1	1.0	1	4.0	4	(3)	3	2.0	2					2.8	3			
Proline	+	1					?	1	+	1	1.1	1																	+	1		+	1		+	1	0.8	1										
Half Cystine																											*	1			*	1																
Glycine	1.0	1	1.0	1	3.7	4	2.3	2	0.9	1	1.3	1			1.0	1			2.2	2						0.7	1			0.9	1											1.0	1	0.9	1			
Alanine			1.5	1.5	1.0	1	3.9	4			1.2	1			0.8	1			2.0	2					2.3	2			1.9	2	3.1	3	(2)	2	(3)	2	4.3	4	3.3	3 ^u								
Valine			1.1	1	0.8	1	4.2	4			0.6	1	0.9	1					1.0	1	0.6	1							0.9	1			1.1	1	1.0	1	0.8	1					2.2	2	2.4	3 ^u		
Methionine	0.3	1									0.5	1													0.8	1																		0.7	1			
Isoleucine	0.5	0.5	0.5	0	0.8	0.5	1.1	1			0.6	1																																1.9	2	1.5	1 ^u	
Leucine	0.5	0.5	0.9	1	0.9	1	1.9	2			0.8	1																																				
Tyrosine																																															0.4	1
Phenylalanine			1.0	1			2.7	3			1.6	2																																				
Tryptophane							*	1																																								
No. of residues	10	9	12	13		25	4	19	2		4	1	11	5	5	5	5	8	9	16	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	2			
yield (10 ⁻⁴ M)	8	15	12	45	6	23	68	85	265	18	18	44	48	6	30	6	13	17	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	75			

b Mouse γ -chain: Residue no. which homologised to known β chain sequences

Mouse- γ -chain Residue no. which homologised to known β -chain sequences																														
Amino acid	1-8		18-30		31-40		41-59		60-61		62-65		66		67-76		78-82		96-104		105-112		121-132		133-144		145-146			
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b		
Lysine	1.1	1					0.9	1	1.0	1	1.0	1	1.0	1	1.1	1	1.0	1	0.8	1	0.9	1	1.0	1	1.0	1				
Histidine											0.9	1							0.7	1							1.1	1	+	1
Arginine			1.0	1	0.8	1												2.3	2	1.8	2	0.6	1	1.0	1					
Aspartic Acid	1.1	1	1.0	1			2.7	3																						
Threonine	1.0	1			1.0	1															0.8	1	1.3	1	0.9	1				
Serine							3.7	4							0.9	1					0.7	1			0.7	1				
Glutamic Acid	1.9	2	3.3	3	0.8	1									0.9	1											2.1	2		
Proline					+	1	+	1												+	1					+	1			
Half Cystine																														
Glycine			2.9	3			1.9	2			0.8	1			1.1	1					0.8	1			0.9	1				
Alanine	1.0	1	0.8	1			1.8	2			0.7	1			0.9	1							4.7	4	3.3	3				
Valine	1.0	1	3.0	3	1.7	2			1.0	1					0.9	1			0.9	1					2.7	3				
Methionine							0.6	1													0.7	1								
Isoleucine							0.7	1							0.9	1														
Leucine			0.9	1	1.8	2	1.0	1							0.8	1	1.8	2	0.8	1	1.5	2			1.5	1				
Tyrosine					+	1	+	1																			+	1		
Phenylalanine	0.9	1					4	2							1.0	1			+	1				1.7	2					
Tryptophane					*	1																								
No. of residues	8	13	10	10	19	2	4	1	10	5	9	8	12	12	2															
yield (10 ⁻⁴ M)	5	4	2	2	10	2	4	3	10	2	3	2	5	4																

c Mouse α -chain: Residue no. which homologised to known β chain sequences

House Y-z chain: Residue No. which homologized to known p chain sequences																											
Amino acid	1-8		31-40		41-59		60-61		62-65		66		67-82		83-89		96-104		121-132		133-144		145-146				
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b			
Lysine	0.9	1	1.0	1	1.4	1	0.9	1	0.9	1	1.0	1	1.1	1			0.8	1	0.8	1	1.1	1					
Histidine	0.7	1							1.1	1			1.3	1			1.2	1					1.3	1	0.9	1	
Arginine																	2.1	2	0.7	1							
Aspartic Acid	1.4	1			2.1	2							1.8	2			2.1	2	0.7	1							
Threonine	0.7	1	1.4	1	1.0	1							1.2	1	0.9	1			1.1	1	1.4	1					
Serine					3.2	3							2.0	2	0.9	1											
Glutamic Acid	1.0	1	1.4	1	0.4	1							2.1	2			0.9	1	2.4	2							
Proline			+	1													+	1	+	1							
Half Cystine																											
Glycine					0.4	1			1.1	1			1.3	1	0.8	1							1.5	1			
Alanine	0.8	1			1.6	2			0.7	1			2.4	2	0.9	1			3.9	4	5.5	5					
Valine	0.6	1	1.5	2	0.6	1	1.1	1					0.9	1	1.1	1	1.3	1					2.4	2			
Methionine					0.9	1																					
Isoleucine					1.2	1							1.1	1													
Leucine	1.2	1	2.1	2	3.3	3							1.4	1	1.2	1	1.2	1					1.5	1			
Tyrosine			0.5	1	+	1																				1.1	1
Phenylalanine					+	1							0.5	1	0.8	1	0.8	1	2.2	2							
Tryptophane			*	1																							
No. of residues	8	10	19	2	4	1	16	7	9	12	12	2															
yield (10 ⁴ M)	10	7	7	12	20	10	8	6	8	10	8	5	8	9	10	8	8										

The embryonic ϵ globin chains of mouse and rabbit were separated^{1,2}. The embryonic ϵ chains were digested with trypsin and after separation of the tryptic peptides by fingerprinting, the amino acid composition analysis was carried out^{1,2}. Line 1 indicates the residue numbers that were homologised to known β chain sequences. No peptide characteristic of adult β chains have been found.

a, Residues; *b*, Nearest whole number of residues.

|| Value not certain, short column was switched off later than usual.

* Proline is present.

* Not present, destroyed during hydrolysis.

** Value for Asx too high, probably because of contamination.

†† Peptide no. 133-144B was probably contaminated by peptide 133-144A.

⊙ Presumably N terminal, partly destroyed.

Values in brackets are estimates because of difficulties with the chromatographic separation or integration.

(10/77) and $\epsilon\gamma$ and $\epsilon\alpha$ (12/77). Almost equally distant seem to be rabbit β and rabbit ϵ (13–14/77) and rabbit ϵ as compared with mouse $\epsilon\gamma$ (14–16/77) and $\epsilon\alpha$ (16–18/77).

In summary, our structural data on embryonic globin chains of mammals show the following characteristic features: in all vertebrate species which have been studied^{2–4,5,7}, there are very early embryonic haemoglobins which contain neither α nor β , but β -like ϵ and α -like χ chains. The χ chain is completely replaced during later yolk sac embryogenesis by the adult α chain. The structure of the χ chain of different species (mouse, rabbit^{1,2}, man^{4,5}) seems to be closely related. No obvious homology apart from their β -like character could be detected for the embryonic ϵ chains of mice and rabbits. Surprisingly, $\epsilon\alpha$ and $\epsilon\gamma$ seem to be more different from each other than from the adult mouse β chain. This might be a consequence of a recent crossover event leading to the β and ϵ chain genes in mice¹⁵. The scarce data on the human ϵ chain¹⁶ do not permit a definitive answer. The human data (ref. 17 and A. Yoshida, personal communication) seem, however, to confirm our impression that mammalian ϵ chains are not very far removed from β chains in contrast to χ and α chains. This failure to find common similarities of the different chains suggests that ϵ or β chains have evolved separately in closely related species such as rabbits and mice, or that ϵ and β chain continuously coevolve¹⁵.

The evolutionary stability of embryonic χ chains and the probably independent evolution of different ϵ chains as well as their close similarity to the β chains suggests that the distinctive functional features of embryonic haemoglobins are due mainly to the χ chains¹⁸. The χ chains may therefore contribute essentially to the high oxygen affinity of embryonic mouse haemoglobins¹⁹.

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Predicted distribution of NAD domain among glycolytic enzymes

X-RAY crystallographic studies have indicated that four dehydrogenases^{1–4}, including lactate¹ and glyceraldehyde phosphate⁴ dehydrogenase, contain a central parallel β sheet consisting of six strands flanked by four α helices. This supersecondary structure⁵ is called the dinucleotide fold or nicotina-

mid adenine dinucleotide (NAD) domain since it forms an NAD-binding site at the C terminus of the β sheet. The dinucleotide fold can be considered as two roughly identical mononucleotide domains each containing about 60 residues arranged in an alternating $\beta\alpha\beta\alpha$ sequence and related by an approximate twofold axis⁵. The aromatic specificity site of subtilisin^{6,7} and the flavin-binding site of flavodoxin^{8,9} seem⁵ to be formed by a secondary structure very similar to a mononucleotide domain of the dehydrogenases. Crystallographic studies have shown that the structures of phosphoglycerate kinase^{10,11}, hexokinase¹², adenylate kinase¹³, phosphoglycerate mutase¹⁴ and triosephosphate isomerase¹⁵ also contain β sheets containing at least five strands flanked by at least three α helices. The nucleoside phosphate or, in the case of the mutase and isomerase, the sugar phosphate-binding site for each of these enzymes is either known^{10–12}, or strongly suspected^{13–15} to be located at the C terminus of the β sheet. Differences occur, however, in some of these supersecondary structures relative to the NAD domain of the dehydrogenase family as regards the direction of the β strands¹², the location of the binding site relative to the plane of the β sheet¹², and most significantly, the sequential order or connectivity of the individual strands in the β sheet^{12–14}. Whether these related supersecondary structures represent convergent^{5,12} or divergent evolutionary processes^{4,16}, or both¹⁷, is a topic of active debate.

We have demonstrated¹⁸ that blue dextran–Sephacryl chromatographic columns seem to function as affinity columns specific for proteins whose nucleoside phosphate-binding sites are constructed by the NAD domain or by a supersecondary structure having a connectivity similar to that of the NAD domain and whose apolar pocket is located above the β sheet. If so, such affinity chromatography can be used to predict the presence of such supersecondary structures in proteins whose high resolution crystallographic structures have not been

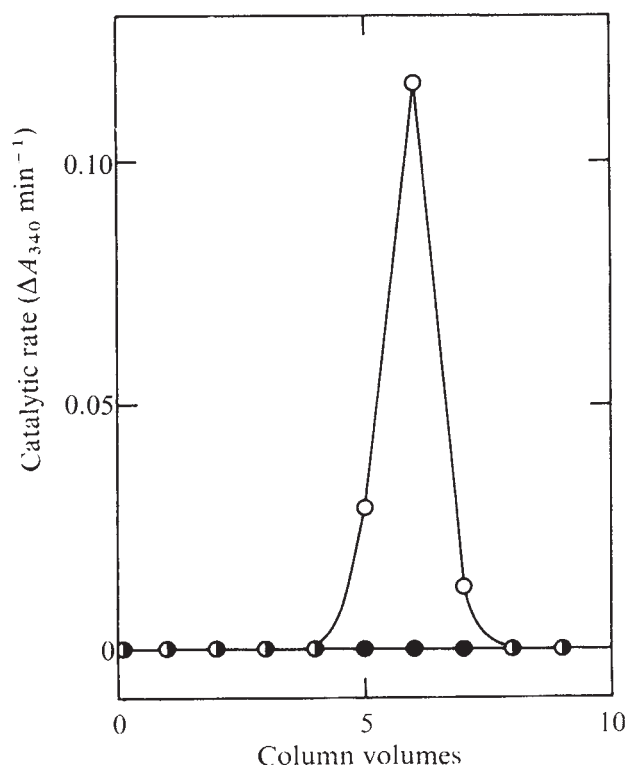


Fig. 1 Elution profile of aldolase from a blue dextran–Sephacryl affinity column. About 100 μ l solution of aldolase containing about 100 μ g protein in 10 mM Tris-HCl buffer, pH 7.5, was applied at zero effluent volume to a 1 ml affinity column equilibrated with the same buffer. The column was then washed with five 1 ml aliquots of the buffer and with five aliquots containing either 10 mM fructose-1,6-diphosphate (○), or 100 mM NaCl (●). Enzymic activity of aliquots of effluent fractions was measured using the standard coupled spectrophotometric assay procedure.

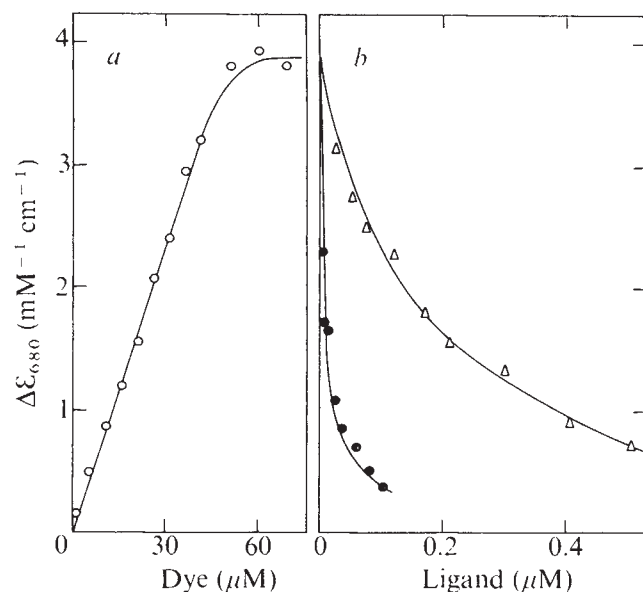


Fig. 2 Spectral measurements of aldolase-dye complexes. *a*, Difference spectral titration of aldolase with dye. Identical aliquots of a concentrated solution of Cibacron blue F3GA dye were added to the same volumes of a 23 μ M aldolase subunit solution in 10 mM Tris-HCl buffer, pH 7.5, and to the solvent. *b*, Difference spectral titration of aldolase-dye complex with ligands. Increasing concentrations of fructose-1,6-diphosphate (●) or NaCl (△) were added stepwise to a 23 μ M aldolase subunit solution in 10 mM Tris-HCl buffer, pH 7.5, containing 77 μ M dye and to a 77- μ M dye solution in the same solvent. Extinction values are in terms of protein subunit concentration corrected for dilution.

reported. Since crystallographic studies have clearly demonstrated that several glycolytic enzymes contain the NAD domain, we have utilised affinity chromatography to predict the distribution of the NAD domain or close analogues thereof among all the enzymes in the glycolytic pathway.

Results of such a chromatographic survey, summarised in Table 1, indicate that phosphorylase *a*, phosphofructokinase, fructose diphosphatase, aldolase and pyruvate kinase specifically bind to the blue dextran-Sepharose chromatographic columns. Each of these enzymes binds to blue dextran chromatographic columns at pH 7.5 and is eluted by a concentration of its specific phosphorylated substrate or effector at least an order of magnitude less than the concentration of the nonspecific ligand, NaCl. A typical elution profile is illustrated in Fig. 1. Although enolase also binds to blue dextran columns at pH 7.5, it is eluted equally well by solutions containing the same ionic strength of specific ligands, phosphoenolpyruvate or 2-phos-

phoglycerate, or the nonspecific ligand, NaCl. Such behaviour is characteristic of ion-exchange chromatography and is not indicative of a specific interaction. By contrast, hexokinase, either in the presence or absence of 10 mM glucose, phosphoglucose mutase, triosephosphate isomerase, and phosphoglucose isomerase do not bind to the chromatographic columns at pH 7.5.

Conclusions as to the formation of specific enzyme-immobilised dye complexes are supported by difference spectral measurements using the free dye of blue dextran, Cibacron blue F3GA. Complexing of the free dye with lactate dehydrogenase and phosphoglycerate kinase produces difference spectra having positive maxima in the range 660–680 nm, presumably due to insertion of the dye into the hydrophobic pockets of the NAD site formed by the NAD domain¹⁹. The hyperbolic dependence of the difference maximum extinction at 680 nm resulting from addition of the free dye to aldolase (Fig. 2) indicates the formation of a discrete enzyme-dye complex. Since the substrate, fructose-1,6-diphosphate, is much more effective than the nonspecific ligand, NaCl, in diminishing the difference extinction (Fig. 2), it is likely that the free dye specifically complexes with aldolase at its substrate sites. By contrast, the nonspecific ligand, NaCl, is equally effective on an ionic strength basis with the ligands ATP and 2-phosphoglycerate in diminishing the difference extinctions of hexokinase-dye and enolase-dye complexes, respectively, indicating that the dye does not bind to the nucleoside or sugar phosphate-binding sites on these enzymes.

These measurements predict that either the catalytic or effector sites of phosphorylase *a*, phosphofructokinase, fructose diphosphatase, aldolase, and pyruvate kinase are likely to be constructed by supersecondary structures closely resembling the NAD domain. The occurrence of such a large common element within the structures of the majority of the glycolytic enzymes suggest that such enzymes may be derived from a common ancestral protein. The predicted occurrence of a structure similar to the NAD domain, in aldolase is particularly noteworthy as this enzyme has no known nucleoside phosphate substrate or effector.

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Table 1 Interactions of glycolytic enzymes with blue dextran-Sepharose affinity columns

Enzyme	Source	Bound	Eluant
Phosphorylase <i>a</i>	RM	Yes	10 mM AMP
Phosphoglucose mutase	RM	No	—
Hexokinase	Yeast	No	—
Phosphoglucose isomerase	Yeast	No	—
Phosphofructokinase	<i>Escherichia coli</i>	Yes	0.1 mM ATP
Fructose diphosphatase	RM	Yes	1 mM AMP
Aldolase	RM	Yes	10 mM FDP
Triosephosphate isomerase	RM	No	—
Glyceraldehyde-3-phosphate dehydrogenase	RM	Yes	10 mM NAD
Phosphoglycerate kinase	Yeast	Yes	1 mM ATP or 3-PGA
Phosphoglycerate mutase	RM	Yes	0.5 mM 2,3-PGA
Enolase	RM	Yes	1 mM 2-PGA or PEP
Pyruvate kinase	RM	Yes	10 mM ATP or PEP
Lactate dehydrogenase	RM	Yes	1 mM NADH

Bound, indicates whether up to 0.5 mg of enzyme binds to a blue dextran-Sepharose affinity column having a 1 ml bed volume equilibrated with 10 mM Tris-HCl buffer, pH 7.5. Eluant, indicates concentration of the listed compound added to the equilibrium buffer which elutes the bound enzyme in at least five column bed volumes.

RM, Rabbit muscle; FDP, fructose-1,6-diphosphate; PGA, phosphoglycerate; PEP, phosphoenolpyruvate. All enzymes were commercially available purified proteins except for *E. coli* phosphofructokinase which was purified as described previously¹⁸. Enzymic activity was measured by standard assay procedures.

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X-ray diffraction patterns from haemoglobin-free erythrocyte membranes

In a survey¹ of X-ray diffraction patterns from erythrocyte membrane preparations produced by hypotonic lysis within a range of tonicities and pH, we emphasised the lability of the haemoglobin-free membrane preparation which readily degenerated during dehydration to give both lipid and residual membrane (lipoprotein) phases. The lipid was identified with a diffraction periodicity of 5–5.5 nm which was sensitive to temperature changes and which could be eliminated by treatment of the membranes with phospholipase C before dehydration². In electron micrographs of sections prepared from the same samples the lipid phase was identified with regions of very fine layering (4 nm periodicity) scattered irregularly through a mass of closely packed layering of much greater dimension, which undoubtedly represented the condensed erythrocyte ghosts. These closely packed ghosts provided periodicities of 20–30 nm, each period including two apposed thicknesses of membrane which were clearly asymmetrical. The corresponding X-ray diffraction patterns also revealed this higher periodicity. We have since experimented with various conditions which can be used to provide haemoglobin-free membranes and have obtained much-improved X-ray diffraction data which confirm and extend our earlier conclusions.

Stomatoff *et al.*³ carried out X-ray diffraction studies from which they concluded that the hydrated, haemoglobin-free erythrocyte membrane is only 5.5 nm thick and is a symmetrical structure. Blaurock and Lieb, in a comment in the News and Views section of *Nature*⁴, remarked on the similarity of the electron density profile proposed for this membrane and the profile of a bimolecular layer of lipids⁵. We have no doubt that the 5.5-nm periodicity analysed by Stomatoff *et al.* does relate to a lipid phase formed in the haemoglobin-free erythrocyte ghost preparation during partial dehydration, and that they have either failed to record or overlooked the higher periodicities which relate to the membrane structure. We have studied membranes prepared exactly as reported by Stomatoff *et al.* and have found their diffraction and electron microscope characteristics to be essentially similar to those of other haemoglobin-free ghosts prepared in hypotonic conditions.

The accumulated data are being analysed in detail in an attempt to elucidate the molecular changes involved in these phase separations which occur during dehydration or partial dehydration of a wide range of membrane preparations. They will be included in a more extensive publication on this topic. Meanwhile it seems important to

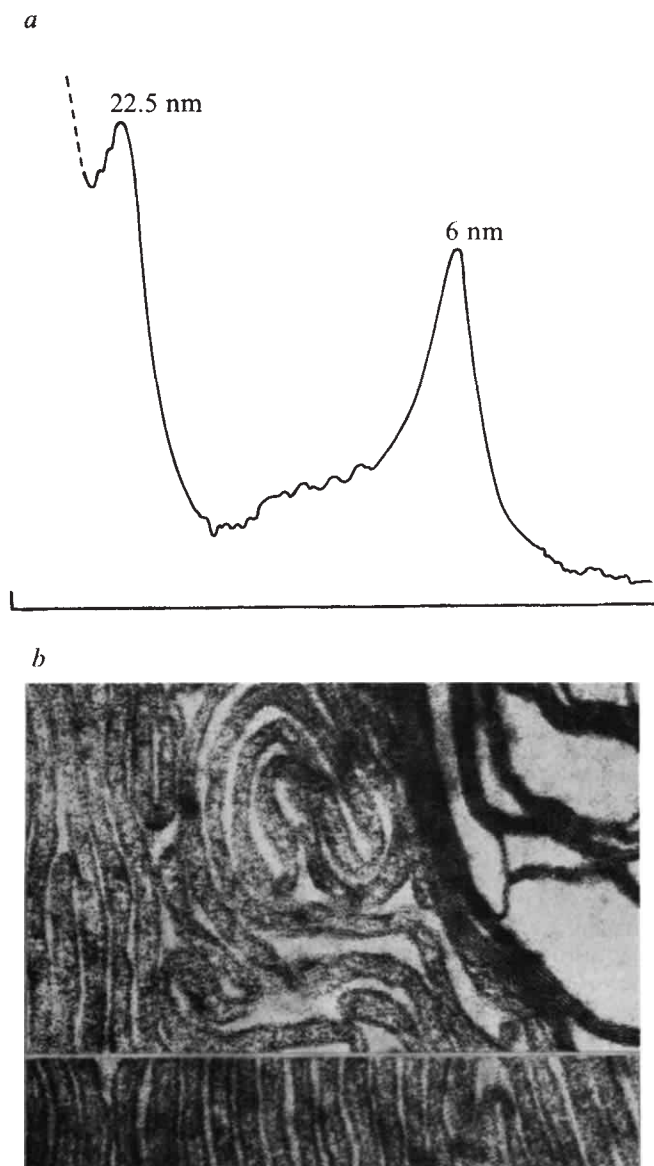


Fig. 1 *a*, Microdensitometer trace of a low angle X-ray diffraction pattern recorded from a haemoglobin-free preparation of erythrocyte ghosts (see text) after partial dehydration. The two principal diffraction bands are identified in terms of the periodicities they represent. *b*, Electron micrograph ($\times 160,000$) from the same erythrocyte membrane preparation, fixed with OsO_4 after partial dehydration, embedded and thin-sectioned. The fine layering on the right is suggested to correlate with the smaller diffraction periodicity and the packing of residual ghosts on the left and below correlates with the large diffraction periodicity.

re-emphasise the significance of this phenomenon in relation to studies of membrane structure by reporting one well-defined example which is particularly relevant to X-ray diffraction studies of haemoglobin-free erythrocyte ghosts.

Figure 1a shows a microdensitometer trace across one of a sequence of low angle X-ray diffraction patterns recorded during the slow dehydration of a sample of haemoglobin-free erythrocyte ghosts which had been prepared (at 0–4 °C) by haemolysis of pig erythrocytes in 20 mosmol bicarbonate buffer, pH 7.0, containing 1 mM EDTA and centrifuged at 200,000g for 3 h. A sample of the wet pellet was enclosed immediately in a chamber maintained at high ($\sim 100\%$) humidity and constant (10 °C) temperature. A sequence of low angle diffraction patterns (limit of resolution 25 nm) was then recorded using a single bent quartz crystal monochromator and a line view of the focus of a rotating anode X-ray generator. Initially

only broad maxima in the region of 6.5 and 10 nm were recorded but as the former intensified and improved in definition an equally intense and well defined reflection was resolved at 2.5 nm. This reduced in successive exposures to about 20 nm and the 6.5-nm periodicity to 5.5 nm in the fully dried sample.

A parallel sample was fixed with osmium tetroxide at an intermediate stage of dehydration (corresponding approximately to the stage represented by Fig. 1a) and prepared for electron microscopy. A representative micrograph is reproduced in Fig. 1b. The bulk of the sample consisted of collapsed erythrocyte ghosts, closely packed to give a periodicity of the order of 30 nm but this was interspersed with small pockets containing material which featured a very fine (~4 nm) periodicity. There is little doubt that the 5.5–6.5-nm periodicity detected by X-ray diffraction relates to this finely layered component which we have previously identified as a lipid phase. The discrepancy in dimension is a consequence of the preparative procedures required for electron microscopy⁶. The residual membranes are responsible for the higher (>20 nm) periodicities. Each membrane features a trilamellar unit with additional dense material at the cytoplasmic face, forming overall an asymmetrical unit which is approximately 10 nm thick in the dried sample. Two such units are apposed in the collapsed ghost.

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State of chlorine and potassium in human platelets and red cells

THE behaviour of excitable cells is consistent with most of their potassium and chlorine being ionised. It has been assumed that this state of affairs is also true of other types of cell. With electron microprobe analysis we have found that most chlorine in human platelets and potassium in human platelets and red cells is present not as free ions but as complexes that can be dissolved by lipid solvents. In platelets, chlorine seems to be complexed with a proteolipid. The anaesthetic halothane, in conditions that will provoke deep anaesthesia, interferes with the complexing between chlorine and the proteolipid in platelets, so that much less chlorine is extractable with lipid solvents.

Air-dried and freeze-dried platelets and red cells had a large chlorine emission peak (Fig. 1) from all parts of their cytoplasm. The microprobe has already revealed an unexpectedly large chlorine peak in muscle¹. By comparing the Cl-S peak ratio with the Cl-P peak ratio, we have attempted to estimate whether the chlorine in platelets is in the membranes or in the ground substance of cytoplasm. Although membranes are present throughout the cytoplasm of platelets, some parts contain more membranes than others. It was assumed that regions with a large phosphorus emission peak were rich in membranes containing phospholipids, for the phosphorus emission peak almost disappears when platelets are treated with chloroform-methanol. The only cytoplasmic region that gives a sub-

stantial phosphorus peak after this treatment consists of the dense bodies that contain stored ATP, ADP and inorganic pyrophosphate². The size of the sulphur emission peak was taken to indicate the thickness of cytoplasm under the beam. We found that the Cl-S ratio varies from platelet to platelet (mean 2.27, s.e. 0.07, $n = 37$) but was relatively constant with each platelet. The Cl-P ratio varied more widely (mean 2.76, s.e. 0.15, $n = 37$). Since the standard error of the Cl-S ratio is so much smaller than that of the Cl-P ratio it seems that the chlorine in platelets is in the ground substance of cytoplasm rather than in the membranes. The reason for the variation in Cl-S ratio from platelet to platelet is not clear.

Virtually all the cytoplasmic chlorine was extracted from desiccated, air-dried or freeze-dried platelets by dry 2:1 chloroform-methanol. Extraction was nearly complete after 1 min and was complete after 15 min (Fig. 1). This was the pattern for normal platelets, for those in which secretion of dense bodies had been inhibited by 0.04 mg atropine per

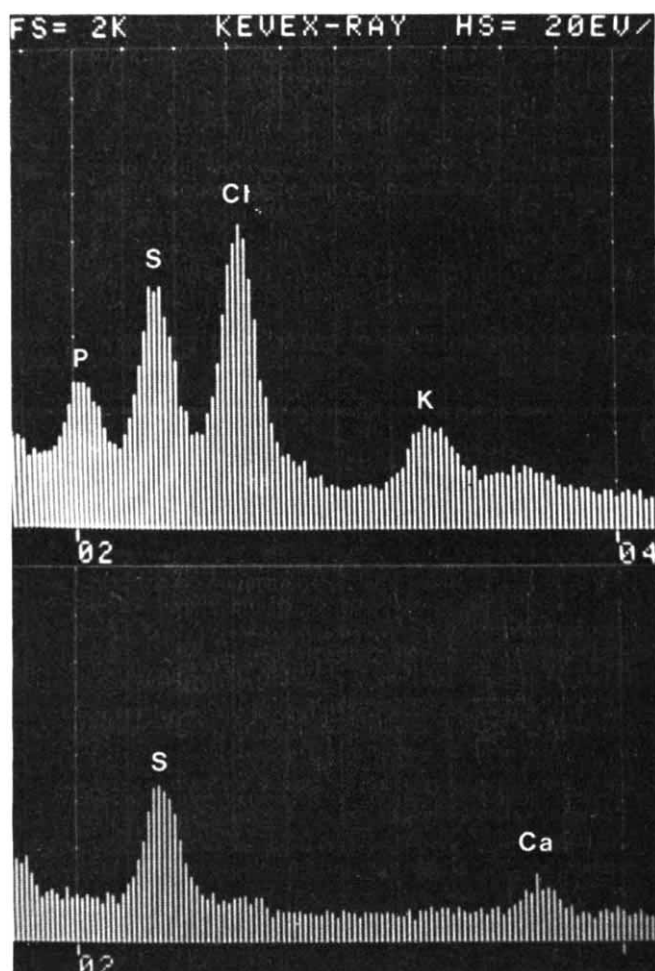
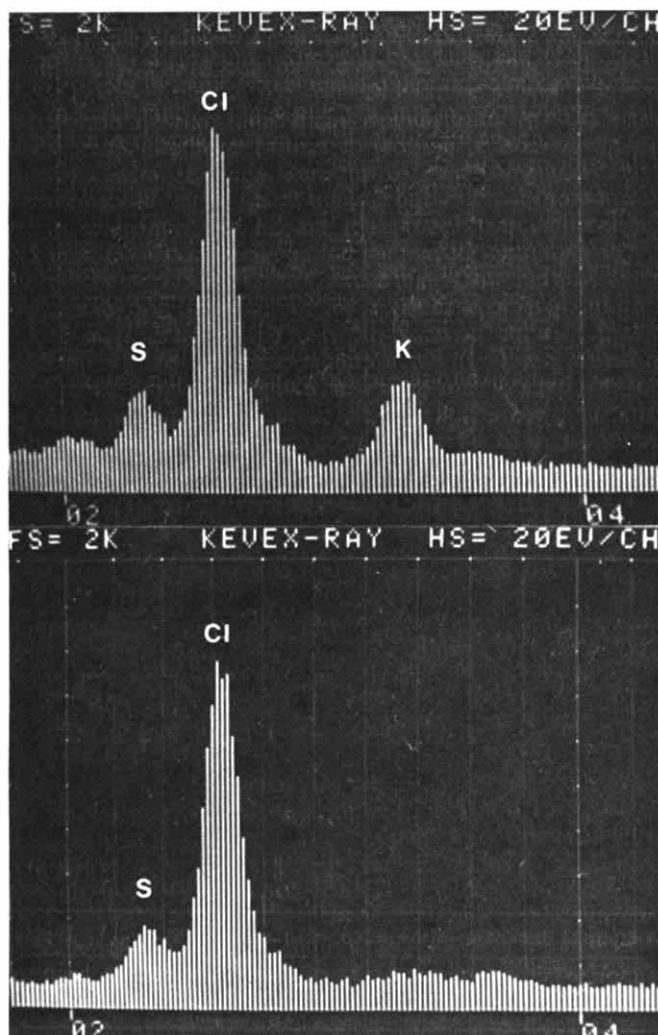


Fig. 1 Emission spectrum from human platelets. The upper spectrum is from the cytoplasm of a normal, air-dried platelet, from a region rich in potassium, the lower from a similar specimen extracted with dry chloroform-methanol. Platelets were prepared from both normal humans and pigs by placing a drop of platelet-rich plasma on carbon-coated copper grids for 10 s and blotting them. They were then either air dried and desiccated for 24 h with silica gel, or immediately freeze dried. For freeze drying the grids were immersed in liquid nitrogen and dried at -60°C in an Edwards tissue dryer Mk 1 for 2 d; they were warmed to room temperature under vacuum. Some grids were extracted for 15 min with 2:1 A R chloroform-methanol prepared from freshly opened bottles and dried with dry Union Carbide molecular sieve 4A (BDH). The specimens were then analysed in an AEI EMMA 4 electron microanalyser equipped with a Kevex Si (Li) energy dispersive detector. A liquid N_2 specimen decontaminator was used throughout this study so that the sulphur peak could be used as standard.

Table 1 Effect of treatment on chloroform-methanol extraction

Treatment applied to living cells	Extractability in chloroform-methanol after drying
Normal pig platelets	All chlorine and potassium extracted
Platelets from pigs anaesthetised with halothane	Varying amounts of chlorine and potassium retained in platelets In many platelets all chlorine and potassium is insoluble
Platelets from pigs stunned electrically at abattoir	Some chlorine is insoluble, nearly half the potassium is insoluble.

ml plasma³, and for those aggregated for 5 min by $2 \mu\text{M}$ ADP at 37°C , before drying. It seems likely that even though removal of water inevitably alters the distribution of charge within a cell, extractability of chlorine by lipid solvents is typical of these cells in life, for the following reasons. First, freeze drying should avoid the creation of high local concentrations of ions that might cause abnormal binding to cell components. No ice crystals were visible by electron microscopy in platelets freeze-dried on carbon-coated grids. Second, various semi-physiological treatments to living platelets influenced the pattern of extractability even after drying (Table 1). The variation in response to halothane was probably due to sequestration of a proportion of the total platelet population in regions where the concentration of halothane was less.

Fig. 2 Emission spectrum from human red cells. The upper spectrum is from a normal, air-dried red cell, the lower from a similar specimen extracted with dry chloroform-methanol.

The chlorine dissolved from platelets by dry 2:1 chloroform-methanol was almost all precipitated from this solution by diethyl ether. This ether precipitate consisted of white fibres, soluble, like the apoprotein of a proteolipid^{4,5} in either dry chloroform-methanol or distilled water. The ability of this complex to exist in either hydrophilic or hydrophobic situations may well be crucial to its functioning in platelets. After storage for a few days at -20°C the dry precipitate lost its solubility in water, and later in chloroform-methanol. The extractability of chlorine from dry platelets by chloroform-methanol also decreased as storage time of dry platelets increased. After prolonged storage no chlorine could be extracted from dry platelets by chloroform-methanol. In aqueous solution the ether precipitate had an absorption shoulder at 280 nm; with silver nitrate solution it gave a thick curdy precipitate shown by the microprobe to contain silver and chlorine. A balance sheet of chlorine present in platelets and recoverable from the proteolipid precipitate is being constructed.

The chlorine in human red cells was almost entirely insoluble in chloroform-methanol (Fig. 2). Red cells provided a useful internal control that demonstrated that platelets do not lose their chlorine through hydration of the chloroform-methanol. Adjacent platelets and red cells on the same grid square lost virtually all or virtually none of their chlorine respectively when extracted with chloroform-methanol.

Potassium had a very uneven distribution in the cytoplasm of platelets. Some regions had three to four times the concentration of other regions. The regions with a high concentration of potassium have not been identified. The potassium of dry platelets and red cells was almost entirely soluble in dry chloroform-methanol (Figs 1 and 2). Since this was as true for freeze-dried cells as for air-dried, this cannot have happened through abnormal binding due to high intracellular concentrations produced by air drying.

Much of the potassium extracted by chloroform-methanol was precipitated by ether as a fibrous precipitate soluble in both chloroform-methanol and in distilled water. It seems possible that, as in the case of chlorine in platelets, complexes with proteolipids influence the ionisation of potassium in red cells. This is thus a special case of the theory that cytoplasmic proteins adsorb water and metal ions co-operatively⁷. Preliminary studies with other cell types (human leukocytes, pig endothelial cells and rat striated muscle) suggest that the pattern of solubility of their cytoplasmic chlorine and potassium in lipid solvents resembles that of the red cells. The availability of free ions in cells may be controlled through complexing with proteolipids. If this were so then the observed action of halothane on platelets may be related to its anaesthetic action on excitable cells.

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matters arising

Interpretation of apparent ages in Minnesota

GOLDICH and Hedge¹ have reported ages from the Montevideo gneiss in south-western Minnesota of $3,950 \pm 70$ Myr. They also obtained an age of 3,800 Myr by including a sample from the Morton Gneiss (54 km distant). The results apparently indicate that those rocks are at least as old as any yet found on Earth. Our work in that area² does not, however, support those results. Evidence that a Rb-Sr age has been inadequately measured is provided by the failure of some of the samples to lie on an isochron (within experimental error). That occurs with two of the samples measured by Goldich and Hedge¹. In addition, the least squares fit for all of their data gave an age of $3,688 \pm 310$ Myr (2σ) and the best fit for their Montevideo Gneiss samples is $3,460 \pm 370$ Myr (2σ). In conditions of partial re-equilibration it is possible to obtain apparent ages which could be either too old or too young.

To understand better the extent of Sr mobilisation during the metamorphism of this area, we have sampled the Montevideo Gneiss on two scales: multiple samples from individual blocks about 2 m in size, and on the scale of kilometres, as was done by Goldich and Hedge¹. Analytical data are given in Table 1. The samples collected from the individual blocks may reveal local, partial or complete equilibration at the time of metamorphism. If that is so, there is no reason to believe that widely separated samples represent anything more than individual points on different local metamorphic isochrons, and their colinearity may well be fortuitous, and the age so indicated erroneous.

The Montevideo Gneiss comprises bands of grey rock interlayered with red bands. Goldich sampled the grey portions to avoid contamination with the younger red material. According to Goldich and Hedge the 3,800 Myr isochron indicates that the grey rock remained a closed system during the emplacement of the red phase (Goldich, personal communication). We do not find that to be the case. The effect of local re-equilibration is seen most clearly in the data from block MV-102 (Fig. 1a). Regardless of any original difference in age, both the grey and red bands lie on a single isochron at $2,472 \pm 44$ Myr (2σ); the high initial ratio, 0.7121 ± 0.0008 (2σ) strongly suggests that it represents a time of meta-

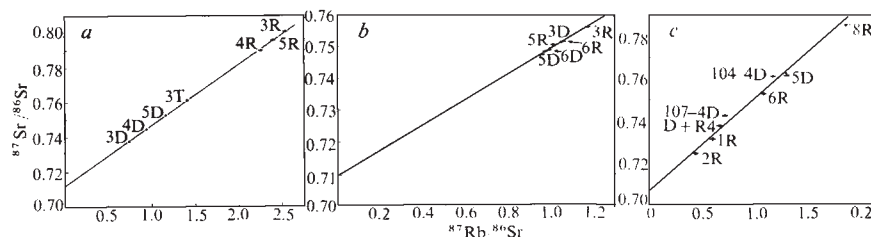


Fig. 1a Rb-Sr isochron for the red and dark bands of block MV-102 from the Montevideo Gneiss (age, $2,472 \pm 22$ Myr (1σ); initial ratio, 0.7121 ± 0.0004 (1σ). Error: 1% in $^{87}\text{Rb}/^{86}\text{Sr}$, 0.0001 (1σ) for $^{87}\text{Sr}/^{86}\text{Sr}$. b, Block MV-100 isochron with red and dark phases from the Montevideo Gneiss near Carlton Lake (age, $2,727 \pm 199$ Myr. (1σ); initial ratio, 0.711 ± 0.004 . c, Best fit isochron for samples from the locality KA-209 of Goldich¹, including the red and the dark phases (age, $3,111 \pm 202$ Myr; initial ratio, 0.706 ± 0.001 . R, Red phase; D, dark phase; T, transitional phase.

morphic re-equilibration, rather than of original crystallisation.

To a lesser but still considerable extent the effects of local mobilisation are observed at the other two sites we have sampled (MV-100 and KA-209). We believe that samples from those localities were among those used by Goldich and Hedge in constructing their 'isochron'. Locality KA-209, the designation of the most radiogenic point obtained by Goldich and Hedge, is presumably the locality previously designated that by Goldich, Hedge, and Stern³.

Results from block MV-100 are given in Fig. 1b. In this case the range of

Rb-Sr ratios is not large; however, all of the points lie well to the right of the isochron of Goldich and Hedge and, again, the dark and red rocks no longer preserve any record of possible original age differences. Samples from locality KA-209 (Fig. 1c) were not from a single block, but from an area about 20 m in dimension. Perhaps for that reason, because of local variability in the extent of remobilisation, the samples were not completely re-equilibrated at 2,500 Myr. Both the dark and red phases scatter along a $\sim 3,100$ Myr 'errorchron'⁴. Our sampling reveals no tendency for either the dark or red phases from any of these

Table 1 Analytical data

Sample no.	$^{87}\text{Sr}/^{86}\text{Sr}$	$^{87}\text{Rb}/^{86}\text{Sr}$	Sr(p.p.m.)	Rb(p.p.m.)	K (weight %/weight)	K/Rb (weight %/weight)
a*						
4R	0.79013	2.2620	256.3	201.5	7.47	371
4D	0.74510	0.9393	258.3	84.3	2.06	244
5R	0.79634	2.4005	291.5	243.2	6.70	275
5D	0.75308	1.1576	256.5	103.2	2.36	228
3R	0.80094	2.5442	262.9	232.6	6.44	277
3T	0.76153	1.4111	249.4	122.3	1.92	157
3D	0.73764	0.73819	278.1	71.4	2.03	284
b*						
3R	0.75595	1.1705	320.9	130.6	3.18	242
3D	0.75150	1.0551	314.9	115.5	1.52	131
5R	0.75055	1.0085	329.7	115.6	2.86	247
5D	0.74835	0.9712	331.8	112.0		
6R	0.75121	1.0437	329.5	119.6		
6D	0.74876	0.9898	335.8	115.5	1.50	130
c*						
MV-9-2R	0.72456	0.4422	501.6	77.1	3.78	490
MV-9-4 D+R	0.73733	0.6662	305.4	70.7	2.39	338
MV-9-6R	0.75256	1.0826	265.8	100.1	3.19	319
MV-9-5D	0.76159	1.2994	204.3	92.3	2.55	276
MV-9-8R	0.78491	1.8580	193.0	124.1	5.45	437
MV-9-1R	0.73087	0.5840	451.3	91.6	5.07	554
MV-104-4D	0.76119	1.1696	211.7	86.0	2.51	291
MV-104-9D	0.76250	1.2918	197.3	88.6	2.42	273
MV-107-4D	0.74266	0.7180	250.6	62.5		

*a, data from MV-102 (Fig. 1a); b, data from MV-100 (Fig. 1b); c, data from KA-209 (Fig. 1c).

localities, taken individually or in combination, to be aligned on a 3,800 Myr isochron. Rather, they seem to be ancient rocks of uncertain age (probably >3,100 Myr), which, locally, have been partially or nearly completely reset during a phase of metamorphism about 2,500 Myr BP. Ages in the vicinity of 3,300 Myr BP are suggested by zircon data^{2,3}.

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¹ Goldich, S. S., and Hedge, C. E., *Nature*, **252**, 467 (1974).

² Farhat, J. S., and Wetherill, G. W., *Abstr. A. Mt. geol. Soc. Am.*, **6**, 729 (1974).

³ Goldich, S. S., Hedge, C. E., and Stern, T. W., *Bull. geol. Soc. Am.*, **81**, 3671 (1970).

⁴ Brooks, C., Hart, S. R., and Wendt, I., *Rev. Geophys. Space Phys.*, **10**, 551 (1972).

GOLDICH AND HEDGE REPLY: We wish to correct a procedural error in our original article¹ relating to the age of $3,950 \pm 70$ Myr (2σ) with an initial ratio of 0.698 ± 0.004 . The use of 2σ is not proper because of the small number (5) of samples. The recalculated results give an age of $3,950 \pm 130$ Myr with an initial ratio of 0.698 ± 0.002 (95% confidence level). We included "sample 339 from the Morton Gneiss as a guide to the initial ratio of 0.700 and an age of 3,800 Myr."

Rb-Sr analyses of the tonalitic phase of the Morton Gneiss of Lund² have progressed to a 7-point isochron with an apparent age of $3,630 \pm 60$ Myr with an initial ratio of 0.6994 ± 0.0004 (95% confidence level). The data will be published in the near future. Additional work is also in progress in the Granite Falls

area. The fact that two points do not lie on the isochron in our original diagram means that those samples have had somewhat different histories and is not evidence that an age has not been measured. In this regard some of Farhat and Wetherill's data are relevant.

Four samples of the 'red phase' from locality KA-209 and three samples from locality MV-100, together with three unpublished analyses by Hedge of the more massive granitic phase, define an isochron (Fig. 1) with an apparent age of $3,000 \pm 90$ Myr with an initial ratio of 0.7065 ± 0.0016 (95% confidence level). Three different localities are represented, but in spite of Farhat and Wetherill's reasoning we feel that the linearity is more than simply fortuitous. The 3,000-Myr age in the Granite Falls area was noted in our original paper¹. It may represent a regional high-grade metamorphic event that affected both gneissic and more massive phases of the Montevideo Gneiss, but it is possible, if not more likely, that it dates the time of intrusion of granitic magma in a foliated terrain of tonalitic to granodioritic rocks. Both phases were later affected by at least two younger events. We are dealing with a complex geological history in which the effects of interaction between magma and country rock as well as regional metamorphism must be considered.

The 2,470 Myr isochron (MV-102) also is useful, and is not unexpected as we have similar unpublished data. The Montevideo Gneiss has undergone intensive shearing and hydrothermal alteration, and some of the apparent ages in the range 1,850–2,500 Myr BP (ref. 4) may be related to this type of activity. We suggest that Farhat and Wetherill have not considered the variety and complexity of the geological processes and that neither the 3,800 Myr isochron¹ nor the 3,000 Myr isochron are necessarily 'fallacious isochrons'³.

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¹ Goldich, S. S., and Hedge, C. E., *Nature*, **252**, 467 (1974).

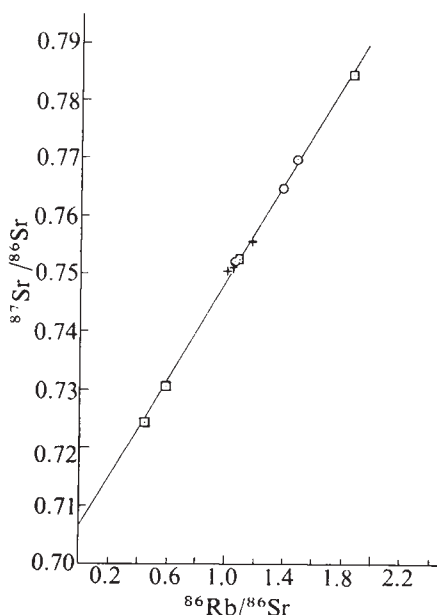
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³ Farhat, J. S., and Wetherill, G. W., *Nature*, **000**, 000 (1975).

⁴ Goldich, S. S., Hedge, C. E., and Stern, T. W., *Bull. geol. Soc. Am.*, **81**, 3671 (1970).

⁵ Goldich, S. S., *Abstr. geol. Soc. Am.*, **4**, (5), 322 (1972).

Fig. 1 Rb-Sr isochron diagram of the massive granitic phase of the Montevideo Gneiss. Localities KA-209 (□) and MV-100 (+) from Farhat and Wetherill¹; (○), new data.



Inhibition of Na, K-activated ATPase and release of neurotransmitters

GILBERT *et al.*¹ have raised the question whether a change in the local environment of the neuronal membrane Na, K-activated ATPase (ATPase) could result in a change both in its activity and in its conformation such that an increase in exocytosis occurs; in this manner the activity of ATPase in the

nerve terminals and the regulation of neurotransmitter release would be coupled. It has also been suggested that the physiological release of acetylcholine from guinea pig ileum and rat brain cortical slices may be mediated through inhibition of the neuronal membrane ATPase^{2,3}. We have shown that several procedures which are known to inhibit ATPase cause a marked release of noradrenaline from sympathetic nerve terminals of the cat spleen^{4,5}. Since physiological release of neurotransmitters is dependent on calcium entry into the neuron^{6,7} the question arises whether calcium entering the neurone during depolarisation may inhibit ATPase⁸, so that inhibition of ATPase is the underlying mechanism in the physiological release of neurotransmitters.

It is generally believed that physiological release of noradrenaline from sympathetic nerve terminals occurs by exocytosis. This belief is mainly based on the demonstration of proportional release of noradrenaline and the soluble form of the vesicular enzyme dopamine- β -hydroxylase (DBH) in response to stimulation of sympathetic nerves⁹. If the physiological release of noradrenaline is mediated through ATPase inhibition, then procedures leading to a decrease in ATPase activity should cause a proportional release of noradrenaline and DBH. We demonstrated however that one such procedure, namely sodium deprivation, caused a pronounced release of noradrenaline (800 ± 85 ng g⁻¹, $n=3$) from cat spleen slices yet the release of DBH over the background level was barely detectable¹⁰. This result indicates that at least noradrenaline release from sympathetic nerves by sodium deprivation is due to some mechanism other than exocytosis. If the mechanism of release of noradrenaline by sodium depletion is presumed to be due to inhibition of ATPase⁴, then physiological release of neurotransmitters by exocytosis is not simply the result of ATPase inhibition.

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¹ Gilbert, J. C., Wyllie, M. G., and Davison, D. V., *Nature*, **255**, 237–238 (1975).

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⁴ Garcia, A. G., and Kirpekar, S. M., *Br. J. Pharmacol.*, **47**, 729–747 (1973).

⁵ Garcia, A. G., and Kirpekar, S. M., *J. Physiol., Lond.*, **235**, 693–713 (1973).

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⁸ Skou, J. C., *Biochim. biophys. Acta*, **23**, 394–401 (1957).

⁹ Weinshilboum, R. M., Thoa, N. B., Johnson, D. G., Kopin, I. J., and Axelrod, J., *Science*, **174**, 1349–1351 (1971).

¹⁰ Garcia, A. G., and Kirpekar, S. M., *J. Pharmac. exp. Ther.*, **192**, 343–350 (1975).

reviews

AN author with the technical knowledge and presentational skill of John Maddox needs no introduction or recommendation to readers in the field of energy. His experience as science correspondent of the *Guardian*, Editor of *Nature* (1966–73), and his frequent contributions to radio and television on topical scientific subjects fit him well to project himself 'beyond the energy crisis'. Those who have read his earlier work, particularly *The Doomsday Syndrome*, will want to get hold of this new one*.

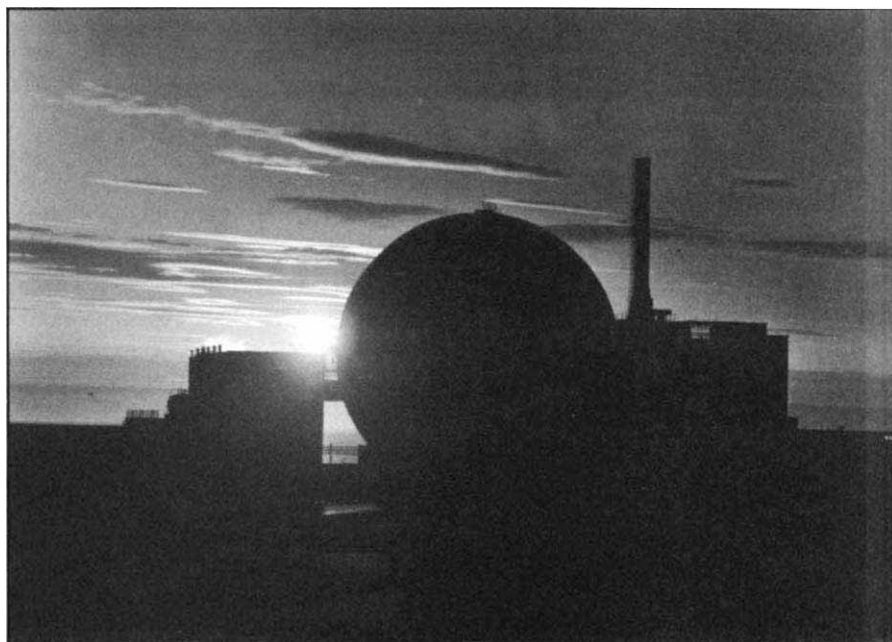
There are no visual illustrations or tables in the book but the words are well chosen and the sentences crisp and vivid. He prepares a launching pad for the main verbal discussion of energy market forces, success by the OPEC oil cartel and the problems of heavily oil-dependent customers by considering the "history" of energy supply and use.

In describing the patterns of energy consumption Maddox ensures that the reader need not be technical to enjoy and understand the presentation. He explains the units for energy and power clearly for the layman and is constantly bringing in something of interest for the specialist—for example, that the Second Law of Thermodynamics was the test advocated by C. P. Snow for the literacy of non-scientists. Both would of course know now about the heavy dependence of many countries on imported oil, but perhaps not the detail of it for specific countries; nor might they have fully appreciated the financial problems arising for many industrial and developing countries alike whose development processes are postponed when oil supply is restricted.

Maddox the realist comes through as he assesses the coal, oil, gas and nuclear energy supply prospects (the new lodes to work) and weighs their advantages and disadvantages. Why not have the best of both worlds and rely instead on new sources of energy free from hazards—solar, geothermal, thermonuclear? The short answer—serious doubts about the cost and the feasibility of meeting growing demand for energy by these means. He concludes that the contribution they will make to future energy supplies will be negligible for several decades and may always be small.

When discussing the policies fol-

UKAEA



Rising to the occasion

lowed by the energy hungry, advanced or industrial countries Maddox asks why their governments should have been so naive in failing to follow the precepts of elementary economic textbooks, and concludes that they simply did not look far enough ahead to protect their interests against an oil supply cartel or shortage. He criticises their foolishness in not having seen the signs, for example, from events in Iran during the Moussadegh period 1952–54, but he is a bit unfair to say that the British decision to initiate the 1955 nuclear power programme because of the fear for the security of oil supplies was wrong. With hindsight it should have been doubled again by 1960 while sticking with the Magnox system, with which design and construction experience had been gained; and again in 1965 with only one Advanced Gas-cooled Reactor Power Station innovation being allowed as a lead into the commercial field. The early Arab Israeli wars were a sufficient pointer to the necessary British energy strategy.

Variation in tempo is a feature of the book, especially as the international monetary problems of today are left behind. The cost of and benefit from insulating a house in Britain enters the discussion. In suggesting that it could be much better to import oil, even at OPEC prices, than to improve the efficiency of energy consumption

Maddox fails to put a value on independence from a cartel which might act again for purely political reasons.

Looking beyond the energy crisis to the coming decades the realist Maddox again emerges, seeing it as unthinkable that Britain would consider the unemployment that a sharp decrease in energy consumption would cause more acceptable than the small risks of reactor accidents; just as developing countries would not settle for slower economic growth or reduced agricultural output for the sake of keeping nuclear power at bay.

Clearly, there is an interesting way ahead, with the possibility that the oil consumers might reduce their oil consumption by 10 to 15% to bring the divergent interests of OPEC members to the surface and/or diversify their energy resource investments, or apply import duties and quotas as a means for encouraging a more equitable, lower price to be set for oil. Clearly, the energy crisis is not over; how can it be when our industrial revolution of more than a century ago is still spreading round the world with a voracious energy appetite. The oil crisis is today, the uranium crisis may be tomorrow; both are seen by Maddox as "merely occasions for the exercise of that blend of courage and realism on which industrial society is founded."

G. R. Bainbridge

* *Beyond the Energy Crisis*. By John Maddox. Pp. 208. (Hutchinson: London, 1975.) £3.95.

Plant and leaf

Phytochrome and Photomorphogenesis: An Introduction to the Photocontrol of Plant Development. By Harry Smith. Pp. xiii+235. (McGraw-Hill: London and New York, 1975.) £7.95.

THE quotation from Stephen Hales and the coloured frontispiece illustrating light- and dark-grown seedlings are a fitting introduction to the contents of this book. In the nine chapters that follow the author has analysed how light from particular spectral regions is perceived, measured by the plant and translated through modifications of biochemical pathways into a recognisable pattern of growth and differentiation. Apart from relevant discussion of the spectral activity and nature of the receptor for phototropic responses, growth effects dependent on the direction of light (phototropism) or time of exposure to light (photoperiodism) are not within the scope of this book.

Before describing the evidence for the existence of specific photoreceptor molecules the author has included a chapter on the physical properties of light in relation to action spectra and photochemical response with methods for experimental manipulation of light sources. This will be particularly help-

ful to those who are entering this field of photobiology for the first time.

The important experiments leading to the discovery, isolation and identification of a chromoprotein absorbing at red and far-red wavelengths, and the evidence for another receptor absorbing in the blue region of the spectrum, have been carefully culled from the literature, and are dealt with historically and analytically with great clarity. In one chapter the chemical properties of phytochrome are treated in detail, with present views of the likely photo-transformations and conversions of the chromophore-protein complex both *in vitro* and *in vivo*.

The quantitative detection within the plant, the likely localisation within membranes, and the evidence linking developmental responses to different forms of phytochrome *in vivo* is presented in a way that leaves the reader in no doubt of the hard facts as well as the problems and paradoxes.

Major proposals for the mechanism of phytochrome action are discussed on the basis of chemical and biological data. The extensive studies of phytochrome involvement in germination and seedling growth form the subject of two chapters, but here some crispness of presentation is lost amongst the many factual details. The longest chapter is devoted to biochemical changes associated with the phytochrome-mediated photocontrol of development.

The book as a whole is carefully and thoughtfully written and easy to read, its many subtitles providing quick reference from one section to another. As a comprehensive survey of the present state of knowledge in the field, with pointers to areas of future study, this work is valuable for students, teachers and research workers and all who wish to learn about phytochrome in plant growth. An informed interest in photomorphogenesis must surely result for anyone who reads this stimulating book. **Daphne J. Osborne**

The Shoot Apex and Leaf Growth: A Study in Quantitative Biology. By R. F. Williams. Pp. vii+256. (Cambridge University Press: London, March 1975.) £6.50; \$18.95.

THE organisation of leaf primordia at the shoot apex, which in many cases can be described mathematically, is a fundamental and obvious system for studying the concepts of positional information and development of form in plants. It is always surprising to me that the shoot apex has not been exhaustively studied in this respect, compared with the more widely used animal systems. A treatise on the shoot apex and leaf growth is therefore potentially exciting reading to those interested in the development of

form. The bulk of this book, 143 out of 223 pages, is devoted to detailed descriptions, ultimately expressed as three-dimensional scale drawings, of the vegetative growth of a dozen plant species encompassing a wide range of different developmental patterns. The development of the inflorescence of wheat is also described, and the detailed comparison of vegetative and floral apices provides some insight into the control of floral induction. These results are supplemented by a further 22 pages detailing the more complicated aspects of the methods used in these studies. After reading these sections which, in the author's words "many will be content to treat as resource material . . . to check the claims made elsewhere", the potential of the shoot apex as a system for studying the development of form is fully justified. Unfortunately, very little is concluded from this wealth of information, and the author in fact suggests that further "comparative, quantitative morphology of these objects will certainly yield valuable insights in its own right". Perhaps, however, this is the time to put the microtome back on the shelf, and try to formulate the type of "insight" on which this type of analysis may throw some light.

The major conclusion drawn from these studies is the importance of physical constraint on the growth rate of the apex and component parts. Although, with hindsight, this is perhaps an expected dependence, it is well supported by the developmental data from the different types of apex, and tested quite convincingly in a series of experiments involving tiller formation in wheat. The claim that physical constraint may play a part in the genesis of form is less convincing. Whether the physical constraint at the shoot apex is the cause or the result of the phyllotaxis and leaf shape is a moot point. It is disappointing that no attempt has been made to experimentally check this hypothesis.

Preceding these sections on the apical growth studies the book contains an interesting chapter (18 pages) on the quantitative description of growth, indicating the problems involved in analysing the growth of a complex, multicellular system. This chapter sets the scene for the subsequent relatively sophisticated growth analyses of the leaves and apex. The subject of phyllotaxis, and particularly the parameters used in quantitatively describing the phyllotactic systems, are very usefully discussed in an introductory chapter (30 pages), although I found it necessary to refer to the more detailed original treatments of Richards in one or two places.

This book is a very significant contribution in terms of the elegant descriptions and analyses of growth of the shoot apex and leaves, but where do we go from here?

John Ingle

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Image Science: Principles, Analysis and Evaluation of Photographic-type Imaging Processes. By J. C. Dainty and R. Shaw. Pp. xiv+402. (Academic: London and New York, January 1975.) £9.80; \$26.00.

SCIENTISTS are constantly finding new ways of exploiting the immense capacity of photographic film for the recording of scientific data. This book will assist students to join in this enterprise, although it will hardly open their eyes to the modern alternatives to film. It deals with the theory of ideal and real photoreceptors of the photographic type, quantum sensitivity, densitometry and the fundamentals of the analysis of image resolution, image noise and the information content of images. The fundamental derivations are certainly given in a way which enables wider use and could be applied to electrooptic image recording and holographic methods, but the techniques themselves are not explained and are scarcely mentioned in the body of the book.

In its sphere, however, this is a good textbook, helping the reader along by its use of well-chosen diagrams and exercises.

Andrew Holmes-Siedle

Principles and Practice in Modern Archaeology. (Teach Yourself Books.) By David M. Browne. Pp. x+262+11 photographs. (Hodder and Stoughton: London, July 1975.) £1.50.

DAVID BROWNE's new book is a welcome addition to the growing literature on practical archaeology, all the more so since it is written with both the layman and the would-be student in mind.

After a rather heavy start, defining certain archaeological concepts, the author moves easily through the more practical aspects of the discipline dealing first with techniques of discovery, then with excavation and finally with the treatment and analysis of materials from excavation and fieldwork. He writes clearly and with the good sense born of experience.

The layout is straightforward and the reference system excellent, in that it leads the interested reader immediately to the most significant up-to-date works without losing him in a flurry of superfluous detail. A list of the major museums is appended for good measure.

This book is to be highly recommended, it cannot fail to dispel the popular misconceptions which still bedevil the subject. For those taking part in excavations it should be compulsory reading.

B. W. Cunliffe

Biocontrol of Rodents. (Ecological Bulletins No. 19) Edited by Lennart Hansson and Bo Nilsson. Pp. 306. (Swedish Natural Science Research Council: Stockholm, 1975.) 45 Skr.

THIS bulletin presents the papers from the Nord-Mus symposium on rodents and forest damage held at Lammi, Finland, in 1974. The early papers review the problems clearly and reveal the severity of the situation especially in monoculture situations.

The greater part of the symposium was concerned with the various app-

Books brief

roaches to rodent control based on the increasing reluctance to add synthetic substances to the environment and the need and wish to control rodents by manipulating the qualities of the environment and the biology of the animals. In addition to being good reviews these papers are a stimulating challenge to biologists to pursue such topics as habitat manipulation, competitive displacement, plant repellence, social mechanisms and the feasibility of genetic control of small mammals. Such work will need a multidisciplinary approach and considerable resources. It is to be hoped that the effort and forward looking nature of this symposium will be rewarded with the assistance it deserves.

Graham Twigg

Engineering in Medicine. By B. McA. Sayers, S. A. V. Swanson and B. W. Watson. Pp. vii+103. (Oxford University: London, May, 1975.) £3.25.

THIS is a curious book but I am afraid it does not quite live up to the description on the cover: "a selective review. Using this as a basis, the authors then discuss prospects and priorities—addressing the problem of identifying developments that should be supported . . ."

I have great sympathy with the authors: everyone in the bioengineering field has opinions on the future of bioengineering and of bioengineers; but if the main part of the book is intended to give an authority to the views contained at the end, apparently addressed to the health departments, it does not succeed very well. I think it is unfortunate that they made the review so wide because, even when one knows the difficulties involved and accepts this explanation, the uneven treatment is still disconcerting. Their technique of condensing infor-

mation has led them to use a profusion of expressions such as "clearly", "on the other hand", and "of course" which is also unfortunate in a book of this type.

It is simply not possible to determine for whom this book has been written. It seems to have been put together as a background book for first year students but someone has thought that it was too difficult and one chapter has more than forty phrases in parenthesis in its twenty pages which give explanations in a kindly way ranging from "features of signal shape (waveforms as they are called . . .)" to "fluid outflow (mainly as urine)". This underlies the reviewer's difficulty: if one cannot determine the real aim it is difficult to judge the value; one might hazard a guess, however, that it was not particularly great. It may be, indeed, that the purpose of the book is an expression of personal opinion on scientific management but it is hard to imagine that those able to influence the organisation of research would read right through to the last chapter, which is a pity.

D. C. Simpson

Fracture of Brittle Solids. (Cambridge Solid State Science Series.) By B. R. Lawn and T. R. Wilshaw. Pp. ix+204. (Cambridge University Press: Cambridge and London, August 1975.) Boards £7.20, \$22.00; paper £3.80.

THIS excellent little book offers a comprehensive, readable, and up-to-date survey of studies of the conditions and mechanisms in the fracture of materials. Griffith's concept of energy balance provides the basis and unifying concept of the treatment. A careful consideration of the macroscopic, molecular and atomic contributions to the energy extends the validity of the concept in a most convincing way, not entirely limited by the adjective "brittle".

The authors write from the standpoint of physical metallurgy, and the strength of the book lies in its detailed and convincing treatment of the physical processes involved in fracture. Mathematical formulations based for example, on the theory of elasticity are quoted, not derived, and the book is therefore complementary to the various monographs on fracture mechanics. Much useful information is presented in diagrammatic and tabular form, with comments in the text.

The book should remain a requirement of the serious student of strength of materials for many years.

J. W. Craggs

obituary

Sir George Thomson, who died on September 17 at the age of 83, was famous for the discovery of electron diffraction. This classical experiment confirmed the hypothesis of de Broglie, and the basis of Schrödinger's wave mechanics, and it also opened the way for an important new technique, which has become an indispensable tool of solid-state physicists and others.

He shared the 1937 Nobel Prize in Physics with Davisson who, with Germer, had seen electron diffraction independently of Thomson, at almost the same time in 1927. They were led to the discovery by an accident, which they described in their fundamental paper, whereas Thomson, inspired by the new theory, knew that such an effect was likely and set out to find it. In his Cornell lectures, which describe the early experiments, he omitted, with characteristic generosity, to mention this difference.

His work depended on a full appreciation of the then new theories and of fundamental physics, and also on the skill and caution of a great experimenter. His discovery was not published until it had been confirmed by more checks and tests than many others would have thought necessary. This discovery, and the manner in which it was made, ensure him a place among the pioneers of pure physics, but he always had an eye for the applications.

He saw immediately the possibilities of electron diffraction as a tool, and over the years contributed much to its development; for this he put to good use not only his experimental skill and flair for the technical, but also his mathematical ability, proved by gaining Firsts in Parts I and II of the Mathematical Tripos. Another subject that attracted his interest was aeronautics, with which he became involved during

the First World War, and which remained a major interest for some time afterwards. He served on the Aeronautical Research Committee at the beginning of the Second World War.

In the 1930s he became interested in neutron physics, and, with his eye for applications, took up the problem of nuclear energy as soon as fission had been discovered. A committee under his chairmanship, set up on his initiative, studied the possibilities of slow-neutron fission. When it became clear that the short term possibilities depended on fast neutrons, he became chairman of the new 'MAUD' committee, whose report led to the project being given high priority in Britain, and probably also accelerated the pace of work in the United States.

After the Second World War, another fundamental field of physics with potential applications aroused his interest. He started to study plasma physics as a means of reaching the high temperatures required for a controlled fusion reaction. This hope has not yet been realised by anyone, but Thomson and his collaborators made important contributions to the early stages of the work.

He was the son of J. J. Thomson, the discoverer of the electron, the founder of a great school of physics in the Cavendish Laboratory, and a great personality. It is often difficult to follow such a father in his own profession, but 'G.P.' did not seem conscious or embarrassed by being so overshadowed. This was no doubt due to his attitude, which was completely lacking in self-consciousness, but was governed by a warm interest in other people and a concern for the essence of the problem in hand. In his many contacts with committees and with administrative problems, there were, of course, occasions when people disagreed with

him, but they were always able to respect his point of view, and the dominant image that one took away from any encounter with him was of absolute integrity, warm humanity, and frank, even blunt, talk. His action in sharing his Nobel Prize with the technician who had been involved in the discovery is characteristic not only of his decency, but of his belief in the importance of technical innovation for the progress of the subject.

One might well apply to him a comment he made when writing about his father: "He was by no means the conventional scientist guided always by logic—if indeed such a creature exists. He was guided by intuitions, not always without prejudice."

George Paget Thomson was born in 1892, educated at the Perse School and Trinity College, Cambridge, where he obtained Firsts in the Mathematical Tripos, Parts I and II, and in Natural Science Part II. His teaching at Corpus Christi College was interrupted by the First World War, when he served in France, transferring to the Royal Flying Corps. He returned to Cambridge in 1919 and was Professor of Natural Philosophy in Aberdeen from 1922 to 1930; he moved to the chair of physics in Imperial College where he remained until he became Master of Corpus in 1952. He retired from that post in 1962, but remained active in many capacities to the end. Among his numerous honours were his knighthood in 1943, the Hughes (1939) and Royal (1949) Medals of the Royal Society, the Faraday Medal (1960) and the presidency of the Institute of Physics (1958–60) and of the British Association (1960).

After the tragically early death of his wife in 1941 he always found time, among his heavy commitments, to remain close to his four children.

announcements

International meetings

December 12–17, **Water resources: water for human needs**, New Delhi (C. V. J. Varma, Indian National Committee for International Water Resources Association, Central Board of Irrigation and Power, Kasturba Gandhi Marg, New Delhi 110001, India).

November 20, **Energy transfer in photo-synthesis**, London (Dr G. S. Beddard, Davy Faraday Research Laboratory, The Royal Institution, 21 Albemarle Street, London W1X 4BS).

December 9–11, **Second international conference on electrical safety in hazardous environments**, London (Anne-Marie Cunningham-Swendell,

The Institution of Electrical Engineers, Savoy Place, London WC2R 0BL).

December 29, **Gordon Research Conference: Metals and metal binding in biology**, Santa Barbara (Dr A. M. Cruikshank, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, Rhode Island 02881).